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Regulation of CD8+ T cell responses to Toxoplasma gondii

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Abstract
Protective immunity to Toxoplasma gondii, an intracellular protozoan parasite, is characterized by a strongly polarized Th1-mediated immune response that is dependent on CD4+ and CD8+ T cells. In order to study the factors that influence development of CD8+ T cell responses to this parasite, a system was developed using a replication-deficient parasite that expressed the model antigen ovalbumin (OVA). These initial studies revealed that an OVA-specific CD8+ T cell response was induced and peaked at day 10 post-immunization, that the primary response was CD4+ T cell-dependent, and that the cells induced by immunization primarily resembled effector cells. Protective immunity to rechallenge using this model was mediated by CD8+ T cells. Long-term study of the CD8+ T cell response was undertaken, in WT mice as well as in c-Rel-/- mice, which are susceptible to T. gondii infection. In these studies, c-Rel was required for optimal primary expansion of CD8+ T cells in response to T. gondii. Surprisingly, while T cells express c-Rel, it was not intrinsically required by the T cells themselves, since adoptive transfer of c-Rel-/- CD8+ T cells to WT mice restored their expansion. Further examination revealed that the inflammatory environment but not the T cells themselves required expression of c-Rel, because exogenous IL-12 rescues the CD8+ T cell responses in c-Rel-/- mice. Maintenance of memory CD8+ T cells as well as secondary expansion of these cells following challenge was independent of c-Rel. This work provided the first characterization of an antigen-specific memory CD8+ T cell response to non-replicating strain of T. gondii as well as showing that c-Rel is not intrinsically required by CD8+ T cells for expansion or effector function following infection, and provides new insights into the requirements for memory cell formation.

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ABSTRACT

REGULATION OF $CD8^+$ T CELL RESPONSES TO *TOXOPLASMA GONDII*

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Protective immunity to *Toxoplasma gondii*, an intracellular protozoan parasite, is characterized by a strongly polarized Th1-mediated immune response that is dependent on $CD4^+$ and $CD8^+$ T cells. In order to study the factors that influence development of $CD8^+$ T cell responses to this parasite, a system was developed using a replication-deficient parasite that expressed the model antigen ovalbumin (OVA). These initial studies revealed that an OVA-specific $CD8^+$ T cell response was induced and peaked at day 10 post-immunization, that the primary response was $CD4^+$ T cell-dependent, and that the cells induced by immunization primarily resembled effector cells. Protective immunity to rechallenge using this model was mediated by $CD8^+$ T cells. Long-term study of the $CD8^+$ T cell response was undertaken, in WT mice as well as in c-$Rel^{-/-}$ mice, which are susceptible to *T. gondii* infection. In these studies, c-Rel was required for optimal primary expansion of $CD8^+$ T cells in response to *T. gondii*. Surprisingly, while T cells express c-Rel, it was not intrinsically required by the T cells themselves, since adoptive transfer of c-$Rel^{-/-}$ $CD8^+$ T cells to WT mice restored their expansion. Further examination revealed that the inflammatory environment but not the T cells themselves required expression of c-Rel, because exogenous IL-12 rescues the $CD8^+$ T cell responses in c-$Rel^{-/-}$ mice. Maintenance of memory $CD8^+$ T cells as well as secondary expansion of these cells following challenge was independent of c-Rel. This work provided the first

characterization of an antigen-specific memory $CD8^+$ T cell response to non-replicating strain of *T. gondii* as well as showing that c-Rel is not intrinsically required by $CD8^+$ T cells for expansion or effector function following infection, and provides new insights into the requirements for memory cell formation.

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Chapter 1 : Literature Review

Introduction

Since *Toxoplasma gondii* was initially described as a pathogen of rodents in 1908, there has been steady progress in understanding the different developmental stages involved in its complex life cycle. Particularly important was the recognition that this parasite can infect any warm-blooded vertebrate. Though congenital infection was the first method of transmission to be understood, additional methods of transmission of *T. gondii* were clarified when it became evident that infective forms of the parasite could be found in the tissues of animals as well as in the environment. Initial descriptions of the ability of *T. gondii* to cause congenital disease highlighted its public health importance and this was reinforced by the recognition that *T. gondii* was an important opportunistic pathogen in immunocompromised patients. In particular, the susceptibility of patients with primary or acquired immune defects in T cell function highlighted the importance of these lymphocytes in preventing reactivation of persistent latent infection. This in turn led to intense study using animal models that defined the events that result in protective immunity, including the important role played by $CD8^+$ T cells and the cytokine IFN-γ in the long-term resistance to *T. gondii*. The studies presented here begin to clarify some unanswered questions about the development of a $CD8^+$ T cell response, and the maintenance of a memory population, that are critical for protection against *T. gondii*. This section of my thesis will discuss the biology of the parasite, the host innate immune

response it provokes, and the main factors that lead to the development of a protective immune response.

1.1 *Toxoplasma gondii*

Toxoplasma gondii is an intracellular protozoan parasite of the phylum Apicomplexa, a group that includes *Plasmodium, Sarcocystis*, and *Cryptosporidium*, and infection with these parasites results in considerable human and animal morbidity and mortality worldwide. *T. gondii* was first identified in a North African rodent called the gundis over 100 years ago, by researchers in Tunis (Nicolle and Manceaux, 1908), and coincidently described in the rabbit by others in Brazil (Splendore, 1908). It was not until 1939 that *T. gondii* parasites were first recognized in humans, specifically in the brain of a congenitally infected infant with encephalomyelitis (Wolf et al., 1939). Parasites isolated from this patient's tissue could be transferred back into mice, rabbits or rats where they replicated and caused disease. Since that time there has been a growing appreciation of the public health importance of *T. gondii*, as well as its usefulness as a model organism to study various aspects of the biology of other Apicomplexa including *Plasmodium*. In this thesis I will explore the regulation of the $CD8^+$ T cell response against *T. gondii*, discuss the importance of this immune cell subset in the initial response to infection, and describe the implications of these studies for memory formation and vaccine development.

Figure 1. Life Cycle of *Toxoplasma gondii*

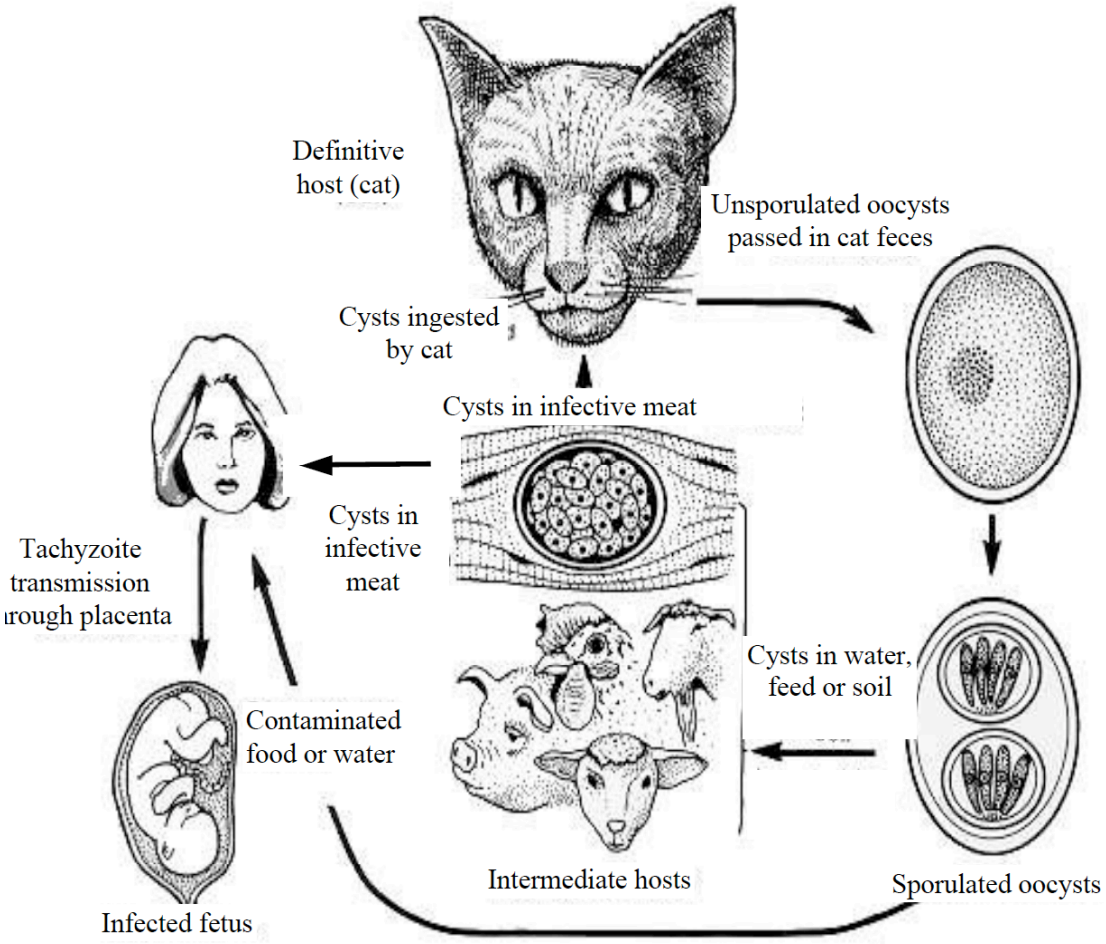


Figure 1. The definitive host of *Toxoplasma gondii* is the cat. Cats shed infective oocysts into the environment, where they are transmitted to intermediate hosts (any warm-blooded mammal, including humans). The oocysts develop into rapidly-multiplying tachyzoites. This form of *T. gondii* can cause tissue destruction and also be transmitted to a developing fetus. Tachyzoites eventually localize to muscle tissues and the CNS where they convert to bradyzoites and persist for the life of the infected host.

Adapted from Medical Microbiology, 4th edition.

1.1.1 Life cycle

The ability of *T. gondii* to survive in many different species throughout a variety of climates is a consequence of many different factors in its life cycle (Figure 1). It has several developmental stages (oocysts, tissue cysts) that facilitate transmission from host to host, or persistence in the environment in a latent form. It can also be transmitted during an acute infection from mother to fetus due to the ability of the tachyzoite form to cross the placenta (Cowen and Wolf, 1950). However, a relatively recent large-scale study of *Toxoplasma* infection in Europe has suggested that the major risk factor for infection is consumption of undercooked or improperly cured meat (Cook et al., 2000).

T. gondii can undergo either asexual or sexual reproduction, though sexual reproduction only occurs in its definitive felid host (Figure 1) and thus will not be discussed in depth any further for the purposes of this thesis. Unlike other protozoa, for example the malaria parasite, the sexual phase of the *T. gondii* life cycle is not required, and serves to enhance the diversity of the species. As noted above, there are two infectious forms of *T. gondii*. Oocysts are the product of sexual recombination in the cat and contaminate the environment, while bradyzoites are found within tissue cysts of chronically infected animals. Following ingestion of either of these infective cysts, *T. gondii* undergoes rapid transformation into tachyzoites. This stage of the parasite can enter nearly any nucleated cell of the host in an active invasion process that is driven by an actin/myosin-based motor complex (Carruthers and Boothroyd, 2007). Following attachment to the outside of a target cell, *T. gondii* tachyzoites then inject the contents of

specialized secretory organelles called the rhoptries (Dubremetz, 2007), allowing formation of a protective double-membraned compartment called the parasitophorous vacuole (PV). This PV is made up of parasite components but also host cell membrane (Melo and de Souza, 1997; Suss-Toby et al., 1996). Within this vacuole the parasite replicates rapidly via the process of endodyogeny, the internal budding process by which parent cells produce two daughter cells (Goldman et al., 1958). This process eventually leads to host cell lysis (Dzierszinski et al., 2004).

It is not yet clear how parasite dissemination from initial points of infection to distal sites occurs, and though extracellular tachyzoites have been found in the bloodstream (Unno et al., 2008), there are also data to support the concept that dissemination occurs via infected cells. For example, phagocytes harboring single parasites were found in the blood of experimentally infected mice, and could rapidly traffic to the brain (Courret et al., 2006). Additionally, adoptive transfer of tachyzoite-infected DC enhanced parasite dissemination, when compared to mice infected with free parasites, in a process that was dependent on G-protein coupled receptor signaling (Lambert et al., 2006). Thus, the acute infection stage of *T. gondii* is characterized by parasite replication and dissemination, and the accompanying development of anti-parasite host immunity. This in turn leads to stress on the parasites and the formation of latent bradyzoite-containing cysts that characterize the chronic stage of infection and allow the parasite to persist.

The transition from tachyzoite to bradyzoite *in vitro* has been linked with a number of environmental stressors including temperature and pH (Soete et al., 1994;

Weiss et al., 1994), but *in vivo,* immune stresses such as nitric oxide (NO) are probably the relevant stimuli. Accompanying developmental stages of the parasite is a specific gene expression profile related to its metabolism and morphology (Dzierszinski et al., 2001; Dzierszinski et al., 2004; Kibe et al., 2005). There are several potential explanations for this change in gene expression, including evasion of the host immune response by the parasite, as studies found that expression of surface antigens differs between tachyzoites and bradyzoites (Kim and Boothroyd, 2005; Kim et al., 2007). Adaptation by *T. gondii* to its new environment is another potential explanation, since the parasite's metabolism differs drastically between the lytic versus latent phase. Regardless, this differentiation within the host to the latent bradyzoite stage is required for long-term persistence and current paradigms suggest that the majority of healthy chronically infected individuals, animal or human, exhibit no clinical indication of disease.

1.1.2 *T. gondii* population structure

As a result of its ability to undergo sexual recombination in the cat, there is the potential for *T. gondii* to generate high levels of genetic variation. However, analysis of the population structure of *T. gondii* has revealed that this organism can be grouped into three clonal lineages, with each of these lineages demonstrating variations in gene expression, epidemiology and virulence (Howe et al., 1997). Conflicting reports exist as to which strains most commonly cause disease in humans, but based on well-accepted animal models it is generally agreed that Type I (typified by the RH strain) are virulent, while Type II (typified by the Prugniaud strain, "Pru") and Type III strains are

considerably less so (Boothroyd and Grigg, 2002; Fuentes et al., 2001; Howe et al., 1997; Khan et al., 2005). Genetic approaches developed in the last 20 years have lead to mapping of virulence factors and shown that virulence can be attributed, in part, to the secreted proteins ROP16 and ROP18, which get secreted into the host cell cytoplasm following invasion (Saeij et al., 2006; Taylor et al., 2006). The majority of the work for this thesis was undertaken using a modified Type I strain that had lost the ability to replicate *in vivo*, and could not form cysts. In addition, studies were undertaken by this laboratory to compare the generation of $CD8^+$ T cell responses against Type I vs. Type II strains and these studies will be further discussed in Chapter 3 and the discussion section.

1.2 Public health importance and vaccine prospects

T. gondii, together with *Salmonella* and *Listeria,* causes the majority of deaths due to food-borne illnesses in the United States and is thus a major public health concern (Mead et al., 1999). Antibody prevalence studies have suggested that anywhere from 10-20% of the U.S. population is chronically infected with *T. gondii* (Jones et al., 2003), though normally this infection is asymptomatic in healthy individuals. Significant clinical disease can occur in individuals that are immunocompromised as a consequence of acquired immunodeficiency caused by HIV, cancers such as Hodgkin's lymphoma, or because of specific treatments designed to suppress the immune system (Ferreira and Borges, 2002). For example, while most humans are capable of mounting appropriate T cell responses required to control *T. gondii*, the decline in $CD4^+$ T cell numbers and loss of $CD8^+$ T cell function seen during AIDS can result in reactivation of latent *T. gondii* and the development of life-threatening Toxoplasmic encephalitis (Luft et al., 1984). In

North America, while Type II strains of *T. gondii* are most prevalent in humans, disease is more commonly associated with Type I strains (Grigg et al., 2001; Howe et al., 1997). While alterations in immune status can profoundly alter the outcome of infection, differences in the parasite population structure can also have a significant impact on the ability of these organisms to cause disease. This is illustrated by recent reports that have noted atypical strains of *T. gondii* associated with ocular disease or acute lethality in immunocompetent individuals (Demar et al., 2007; Grigg et al., 2001; Khan et al., 2006a).

Current drug regimens of sulfadiazine and pyrimethamine, though highly effective against rapidly replicating tachyzoites, are unable to target the bradyzoite stage of *T. gondii* due in part to decreased metabolic inactivity (Luft et al., 1984). Further, while treatment of acutely infected pregnant women can limit the damage caused by replicating parasites, it does not uniformly prevent transmission to the fetus (Thiebaut et al., 2007). Meta-analysis of prenatal treatment against congenital toxoplasmosis has shown that approximately 30% of babies born to infected mothers acquire *T. gondii*, and up to 4% of these babies will die or exhibit permanent neurological defects or visual impairment (Thiebaut et al., 2007). Because of the dangers associated with congenital toxoplasmosis, and because cure of chronically infected individuals is impossible, the development of a vaccine against this parasite is desirable. While a vaccine to prevent *T. gondii*-induced abortions in cattle is commercially available (Buxton and Innes, 1995), it uses a live attenuated strain of *T. gondii* and is therefore unlikely to be approved for use in humans. It has also been proposed that a therapeutic approach would also be useful in the instance of other chronic parasitic diseases, for instance in ameliorating the outcome

of Chagas disease caused by chronic *Trypanosoma cruzi.* Thus, even if the design of a preventative vaccine were not successful, design of a therapeutic vaccine that targets $CD8^+$ T cell responses, or improvements to standard drug treatment, would be helpful to limit the morbidity and mortality associated with clinical toxoplasmosis.

1.3 Model antigens and epitope discovery

Identification of the proteins that drive an immune response to *T. gondii* will not only help to further understanding of immunity to infection, but also provide assistance for rational vaccine design. Protozoan parasites have relatively large microbial genomes compared to most viruses, for example the human immunodeficiency virus (HIV), which contains only 9 genes. Therefore, design of predictive algorithms to identify endogenous epitopes has been met with limited success. Antigen recognition during parasite infection is further complicated by the distinct developmental stages associated with initiation of infection, development of disease, and latency. For instance, *T. gondii* has been shown to express different antigens, depending on the stage of infection, with some antigens being expressed during the fast-replicating tachyzoite stage, and others expressed only when the parasite has encysted in the brain and muscle tissue in its bradyzoite form (Frickel et al., 2008; Kim and Boothroyd, 2005; Kwok et al., 2003; Lutjen et al., 2006).

At the time this thesis work was initiated, no endogenous $CD8^+$ T cell epitopes had yet been identified, so different groups including our laboratory used parasites that expressed the model antigens ovalbumin or beta-galactosidase (Kwok et al., 2003; Lutjen et al., 2006; Pepper et al., 2004). This is a strategy that has been used by many groups to understand immunity to infection. For example, reports using *L. major* and *T. cruzi* that

expressed model antigens suggested preferential processing and presentation of secreted antigens or proteins located on the cell surface (Bertholet et al., 2005; Garg et al., 1997). Similar findings were seen with *Salmonella*, where secreted but not cytosolic antigens were able to induce a potent immune response (Guzman et al., 1998).

Extensive efforts by many different groups have lead to the sequencing and annotation of the *T. gondii* genome (El-Sayed et al., 2005; Gardner et al., 2002; Kissinger et al., 2003), which facilitated the discovery of endogenous $CD8^+$ T cell epitopes. Two epitopes derived from dense granule and rhoptry proteins have been described in BALB/c mice (Frickel et al., 2008), while another endogenous epitope found in B10.D2 mice was derived from a dense granule protein (Blanchard et al., 2008). In *T. cruzi*, a major $CD8^+$ T cell epitope making up to 20% of the total $CD8^+$ T cell pool during acute infection was identified in the trans-sialidase family, a surface and secreted protein (Martin et al., 2006). Thus, in agreement with earlier work using model antigens, the majority of work indicates that parasite-secreted proteins exit the parasitophorous vacuole and enter the endogenous MHC Class I presentation pathway, thus becoming major targets in the immune response to *T. gondii*.

1.4 Overview of Immunity to *T. gondii*

The study of the biology of *T. gondii,* as well as the host immune response it induces, has yielded insight into not only this parasitic organism but also its importance

Figure 2. The immune response to T. gondii

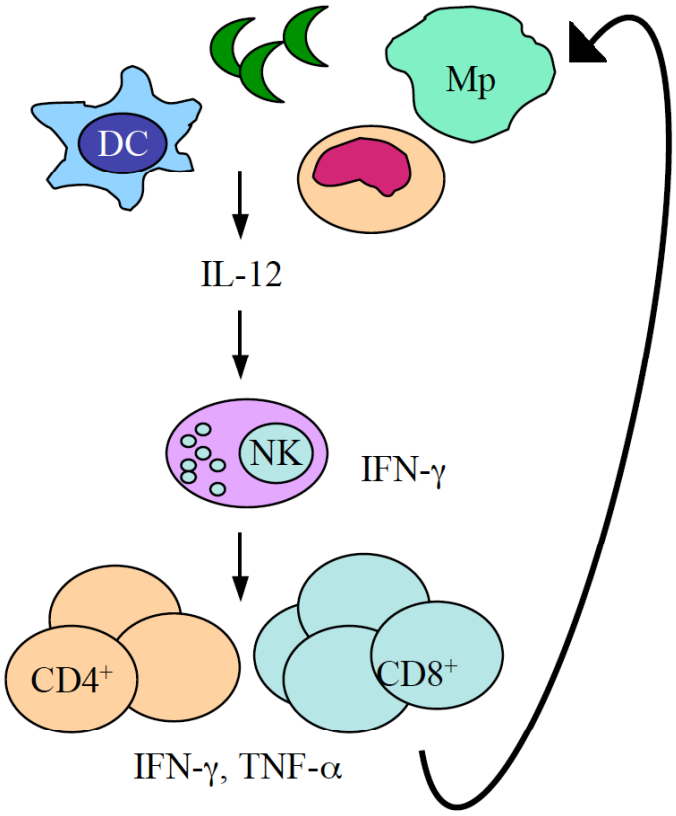


Figure 2. The immune response to *T. gondii* involves acute production of IL-12 from accessory cell populations, as well as production of IFN-γ from natural killer (NK), $CD4^+$ and $CD8^+$ T cells. IFN-γ along with TNF-α acts back on macrophages and induces upregulation of anti-parasitic effector mechanisms that lead to parasite killing and control of acute infection. IFN-γ derived from $CD4^+$ and $CD8^+$ T cells is required to prevent reactivation of latent infection.

as a model organism for other intracellular organisms. The ease by which *T. gondii* can be propagated *in vitro*, as well as ability to genetically manipulate this parasite, have contributed to further knowledge of antigen presentation and immunity induced by the host. The study of infected human patients as well as numerous mouse studies have shown that this organism elicits a strong Th1-type response, characterized by the ability of accessory cell-derived IL-12 to promote generation of parasite-specific $CD4^+$ and $CD8^+$ T cells that produce IFN-γ (Figure 2).

1.4.1 Innate Immunity to *T. gondii*

Following oral infection, the first cells encountered by *T. gondii* are intestinal epithelial cells. In response to infection with *T. gondii* tachyzoites, these cells have been shown to produce cytotoxic molecules like NO as well as chemokines such as MCP-1, MIP-1α and MIP-1β (Mennechet et al., 2002). However, this organism does not remain confined to the intestinal epithelium for very long. As early as 8 hours after oral infection of mice *T. gondii* tachyzoites can be found in the mesenteric lymph nodes (Dubey et al., 1997). It is not well known which cell types are acutely infected by *T. gondii* in the intestine or the lymph node, though it has been suggested that antigen-presenting cells are key targets, and this is an active area of investigation. More is known about the effects *T. gondii* can have on infected cells. Early studies of innate immune responses to *T. gondii* focused on the ability of macrophages to kill intracellular parasites, but our understanding of the pathways that regulate innate recognition to intracellular parasites has expanded immensely in the past two decades. Some of the molecular mechanisms behind these

early events will now be discussed, as will more recent genetic and biochemical approaches to determine how *T. gondii* can directly interfere with these pathways.

Live parasites or proteins isolated from *T. gondii* have previously been used to stimulate individual cell populations such as fibroblasts and macrophages to induce cytokine or chemokine production (Brenier-Pinchart et al., 2000; Butcher and Denkers, 2002). This implies the presence of an innate recognition process that can occur in the absence of adaptive immunity. A main pathway of innate antigen recognition is via the Toll-like receptors (TLRs), an evolutionarily conserved group of receptors that recognizes distinct groups of microbial antigens (or damage-induced host molecules). In fact, several TLRs have been linked with the immune response to *T. gondii* (Gazzinelli and Denkers, 2006) as well as a number of other pathogens (Kawai and Akira, 2007). Most TLRs require the adapter protein MyD88 to transduce their signal, and initial studies in mice lacking this gene revealed that MyD88 was required to prime Th1 responses against vaccination with *T. gondii* antigens (Jankovic et al., 2002). Moreover, *in vivo* studies demonstrated that MyD88 was required to control acute infection with *T. gondii*, similar to what was seen in IL-12p40 KO mice (Scanga et al., 2002), and that the MyD88-KO mice challenged with *T. gondii* had reduced serum levels of IL-12p40 compared to WT mice. Later studies to clarify which TLRs were involved in this response revealed that TLR11 is activated by a profilin-like protein (Yarovinsky et al., 2005). TLR2 was required for survival against high-dose intraperitoneal challenge (Mun et al., 2003) and TLR4 and TLR9 have also been implicated in responsiveness to *T. gondii*-derived antigens in the gut following oral infection (Fang et al., 2008; Minns et al., 2006).

Downstream of TLR activation, two important signaling cascades are induced: NF-κB and Mitogen-Activated Protein Kinase (MAPK), both of which are highly conserved pathways involved in the immune response. The role of NF-κB in immunity will be discussed in more detail in Chapter 1.2. Perhaps not surprising given the complex nature of *T. gondii*, other innate signaling pathways also contribute to recognition of this organism. For instance, the *T. gondii*-derived protein cyclophilin-18 has been reported to activate dendritic cells via interactions with CCR5 (Aliberti et al., 2003), and some studies have demonstrated that CCR5-deficient mice had decreased survival and increased parasite burdens following *T. gondii* infection (Khan et al., 2006b).

Consistent with the idea that these innate recognition events would promote the eventual immune-mediated killing of *T. gondii*, the parasite has evolved multiple mechanisms to interfere with these signaling pathways. For instance, several groups have found that *T. gondii* interferes with NF-κB at the level of subunit phosphorylation (Shapira et al., 2002) and nuclear translocation of this transcription factor (Butcher and Denkers, 2002; Butcher et al., 2001), associated with reduced production of IL-12. Other studies have shown that *in vivo* administration of soluble *Toxoplasma* antigens (STAg) induces a state of refractoriness in dendritic cells, rendering them incapable of producing IL-12 after subsequent pathogen challenge (Reis e Sousa et al., 1999), potentially via its interference with the MAPK pathway (Kim et al., 2004). Further, *T. gondii* is also known to promote sustained activation of STAT3, causing suppression of TNF-α and IL-12 (Butcher et al., 2005b), while at the same time inhibiting nuclear translocation of STAT1 (Luder et al., 2001). The fact that *T. gondii* interferes with multiple signaling pathways

associated with innate recognition and anti-parasitic effector mechanisms indicates the importance of these pathways in limiting early parasite growth and replication.

Numerous studies have indicated that the pro-inflammatory cytokine IL-12 plays a key role in the response to Toxoplasmosis (Gazzinelli et al., 1993; Gazzinelli et al., 1994; Hunter et al., 1995b; Khan et al., 1994). Once IL-12 was recognized as being critical for resistance to *T. gondii*, subsequent studies focused on identifying the source of this cytokine during infection, and a number of innate cell types were shown to produce IL-12 in response to *T. gondii*. A population of inflammatory neutrophils that have the ability to rapidly produce IL-12 has been shown to traffic to the site of infection (Bliss et al., 2000; Bliss et al., 2001). Macrophages are also important in the acute response to *T. gondii* and have been ascribed diverse functions including antigen presentation, IL-12 production, parasite phagocytosis and production of reactive nitrogen intermediates. Dendritic cells, which also produce IL-12 in addition to other pro-inflammatory cytokines, have been shown to play an important role in the immune response to *T. gondii* (Liu et al., 2006). Hours following injection with STAg, IL-12p40-producing DCs can be found in the spleen (Aliberti et al., 2000). The plasmacytoid DC subset has been found to be activated as well as produce IL-12 following *T. gondii* infection, and is implicated in antigen presentation to $CD4^+$ T cells (Pepper et al., 2008).

In addition to skewing the differentiation of naïve T cells toward a Th1 phenotype, IL-12 can also influence the activation and effector function of other cell populations including natural killer (NK) cells, which are an important component of the innate response to *T. gondii.* Very early studies showed that injection of antigen

preparations of *T. gondii* or infection lead to increased NK cell activity (Hauser et al., 1982; Hauser et al., 1983; Sharma et al., 1984). Studies in SCID mice, which lack all B and T cells, demonstrated the expansion of a substantial NK cell response following *T. gondii* infection. This population expanded and produced the IFN-γ required for survival of the acute phase of infection (Hunter et al., 1994; Sher et al., 1993). Mice lacking MHC class I-restricted $CD8^+$ T cells also demonstrated an expansion of NK cells following *T. gondii* infection, and these cells were a considerable source of IFN-γ following *in vitro* restimulation of splenocytes (Denkers et al., 1993b).

The events that lead to the production of IFN-γ have a critical role in promoting resistance to *T. gondii*, and a number of studies document the importance of this cytokine during the acute stage of infection as well as in preventing reactivation of latent disease (Scharton-Kersten et al., 1996; Schofield et al., 1987; Suzuki et al., 1989; Suzuki et al., 1988; Torrico et al., 1991). The importance of IFN-γ in helping control this pathogen has been recognized for more than 30 years, and we continue to gain better understanding of its regulation of anti-parasitic effector mechanisms including reactive oxygen intermediates (ROI) and interferon-γ-inducible GTPases (Collazo et al., 2001; Scharton-Kersten et al., 1997; Taylor et al., 2000). The immunity-related GTPases (or IRGs) are interferon-γ-inducible proteins, at least 6 of which are required for resistance to infection with *T. gondii* (Zhao et al., 2009), and following infection these proteins are recruited to the membrane of the parasitophorous vacuole (Butcher et al., 2005a), but these more recent studies have also highlighted our limited knowledge of parasite killing

mechanisms. This innate response from NK cells, DC and other antigen-presenting cells is a critical link that allows the development of adaptive immunity.

1.4.2 Adaptive Immunity to *T. gondii*

While infection with *T. gondii* elicits a strong innate response that provides an early mechanism of resistance, the long-term control of this organism is dependent on the development and maintenance of parasite-specific adaptive immunity. Challenge with this parasite leads to the sustained production of *T. gondii*-specific antibodies by B cells, and T cells that exert effector functions including cytokine production and cytoxicity. The adaptive immune response has the additional benefit of maintaining memory responses against pathogens it encounters. In this section, we will discuss the role that adaptive immune cells and their effector functions play in the response to *T. gondii*.

Infection with *T. gondii* leads to an expansion of the B cell compartment and subsequent production of parasite-specific antibodies, and it was recognized almost 40 years ago that transfer of sera from immune mice could provide some protection to naive mice (Krahenbuhl 1972). The Sabin-Feldman dye test used for detection and quantification of *T. gondii*-specific serum antibodies is dependent on the ability of antibodies to cause complement-dependent lysis of tachyzoites (Schrieber RD 1980). The role of B cells during *T. gondii* infection was later studied in B cell-deficient (μMT) mice. While these mice were able to survive the primary phase of infection, they succumbed early in the chronic stage (Kang et al., 2000). The ability of μMT mice to gain protection following vaccination was dependent on the parasite strain used to challenge them. While μMT mice could resist a Type II strain they, were not able to

survive challenge with the more virulent Type I strain (Johnson et al., 2004; Sayles et al., 2000), and while transfer of immune sera to μMT mice delayed time to death following *T. gondii* challenge it did not allow them to survive (Johnson et al., 2004). Together, these studies indicate that B cells and antibody contribute to immunity against *T. gondii* by providing a mechanism for parasite killing via antibody-mediated lysis. Nevertheless, B cells are also known to present antigen and produce cytokines, for instance surface TNF-α on B cells can help enhance IFN-γ production by T cells (Menard et al., 2007), but little is known about these other functions of B cells during Toxoplasmosis.

As discussed in the previous section, NK cells provide a limited mechanism of resistance to *T. gondii*. The importance of T cells in the long-term control of *T. gondii* is perhaps best illustrated by the observation that patients with primary or acquired defects in T cell function are susceptible to reactivation of latent bradyzoites and suffer effects in the CNS (Luft et al., 1984). Understanding the contribution of each T cell subset was made possible through experiments using animal models. The transfer of T cells from immunized mice to naïve mice could provide protection against a lethal challenge of *T. gondii*, but this was abolished by depletion of $CD8^+$ T cells prior to transfer (Parker et al., 1991; Suzuki and Remington, 1988). Similarly, transfer of $CD8^+$ T cells from chronically infected mice to naïve WT or nude mice was able to provide protection from *T. gondii* challenge (Parker et al., 1991). These studies were supported by others demonstrating that H2 haplotype profoundly influenced the outcome of infection (Brown and McLeod, 1990; Suzuki et al., 1994), and that $CD8^+$ T cells were the major source of IFN-γ *in vivo* during infection (Gazzinelli et al., 1991; Shirahata et al., 1994). Therefore, a major focus

of this thesis is the generation as well as maintenance of a $CD8^+$ T cell response to *T. gondii*.

As $CD8^+$ T cells are known to exert their effector functions primarily via cytokine production or cytolytic activity, studies were performed to define the contribution of these pathways to control of *T. gondii*. Early reports demonstrated that $CD8^+$ T cells isolated from immunized or infected mice were capable of lysing infected cells or targets pulsed with parasite antigens (Denkers et al., 1993a; Hakim et al., 1991; Khan et al., 1991; Subauste et al., 1991). Initial studies with perforin-deficient mice revealed that they were less susceptible to infection than CD8-deficient mice, and could generate a protective $CD8^+$ T cell response. However, these mice showed increased susceptibility to chronic Toxoplasmosis, indicating that the ability to recognize and lyse infected cells was required for optimal resistance (Denkers et al., 1993b; Denkers et al., 1997). The analysis of cytolytic activity frequently relied on the *in vitro* expansion of rare T cell populations and the use of chromium release assays to demonstrate lytic activity. An *in vivo* cytotoxicity assay has been developed and has proven useful in detecting cytolytic responses induced by viral infection models (Barber et al., 2003) and more recently in the *T. cruzi* infection model (Martin and Tarleton, 2005). Chapter 3 will describe how a similar approach was developed to measure cytolytic activity to *T. gondii*.

Many factors are known to influence the generation of $CD8^+$ T cell responses, including cytokines such as IL-2 and IL-12, which contribute to T cell expansion, survival, and the acquisition of effector function. At the time when these studies were initiated, some work had been undertaken to understand the antigen-specific $CD8^+$ T cell response during chronic Toxoplasmosis (Kwok et al., 2003; Lutjen et al., 2006). In more

general terms, less was known about $CD8^+$ T cell memory responses to most chronic infections. Many parasites form persistent infections for the life of the individual, associated with on-going antigen exposure (Frenkel, 1988; Zhang and Tarleton, 1999). This makes it hard to define memory, as compared to memory $CD8^+$ T cells induced by acute viral infection where the kinetics of pathogen burden and clearance are better defined (Joshi et al., 2007). The phenotype of cells induced *following T. gondii* infection, how this differs from what is seen during acute viral infection, and the consequences in terms of memory formation, will be further discussed in Chapter 3.

$CD4^+$ T cells were demonstrated to play a contributory role in resistance to *T. gondii*, but were not as critical as $CD8^+$ T cells in conferring resistance (Gazzinelli et al., 1991; Parker et al., 1991). Early work focused on the protective role that $CD4^+$ T cells could play in preventing reactivation of latent disease in the brain (Vollmer et al., 1987). $CD4^+$ T cells are the main producers of the T cell growth factor IL-2, and might be playing other helper-like roles for $CD8^+$ T cells or other cell populations during infection. Studies with CD4-deficient mice demonstrated that $CD4^+$ T cells were required to maintain $CD8^+$ T cell responses against chronic *T. gondii*, as demonstrated by a precursor CTL assay (Casciotti et al., 2002). While this thesis work was already underway, the Schlüter laboratory made use of a transgenic parasite to show that $CD4^+$ T cell help contributed to antigen-specific $CD8^+$ T cell function in the brain but not the spleen (Lutjen et al., 2006). Beyond their role in IFN-γ production, it was also known that $CD4^+$ T cells contributed to the antibody response to infection (Araujo, 1991). It is now well understood that that a particular subset of $CD4^+$ T cells called follicular helper cells is

responsible for interacting with B cells to induce their expansion, class switching, and germinal center formation (Kim et al., 2001; Schaerli et al., 2000).

In addition to the protective function they can play during infection, $CD4^+$ T cells have sometimes been shown to play a pathological role during *T. gondii* infection of the central nervous system (Israelski et al., 1989; Stumhofer et al., 2006; Wilson et al., 2005). Part of the difficulty of interpreting the role of $CD4^+$ T cells during *T. gondii* is that different strains of mice demonstrate diverse outcomes, something that has been known for some time (Araujo et al., 1976). Additionally, there are clear instances where $CD4^+$ T cells can play pathological roles in the intestine, dependent on IFN-γ production, which differs between C57BL/6 and BALB/c mice (Liesenfeld et al., 1996). The importance of tight regulation of the immune response to control parasite replication while limiting damage to the host will be discussed in the next section.

1.4.3 Regulation of immune-mediated pathology during *T. gondii*

The immune response to *T. gondii* is highly Th1 polarized and characterized by the systemic production of pro-inflammatory cytokines, and consequently must be carefully controlled to prevent immune-mediated pathology in the host. While all of the factors that contribute to limiting the inflammatory response to *T. gondii* will not be discussed here, a few major findings as they relate to the immune biology of toxoplasmosis will be highlighted. As mentioned above, $CD4^+$ T cells have a complicated role in response to this infection and can contribute to immunopathology, which is partially dependent on host genetic background. Immunoregulatory cytokines,

specifically IL-10 and IL-27, are important in limiting the pro-inflammatory responses of $CD4^+$ T cells and limiting the pathological effects. Early experiments where SCID mice were treated with antibodies against IL-10 demonstrated a prolonged survival of these mice during acute *T. gondii* infection (Hunter et al., 1993), consistent with the idea that IL-10 is antagonist of protective innate responses. However, when T cell-sufficient IL-$10^{-/-}$ mice were infected with *T. gondii*, they experienced a lethal inflammatory response that was driven by overproduction of IFN-γ by $CD4^+$ T cells, as well as TNF-α and IL-12 by splenic macrophages (Gazzinelli et al., 1996). IL-$10^{-/-}$ mice also have increased levels of pro-inflammatory cytokine production and immune pathology in the brain during chronic infection (Wilson et al., 2005). It was later found that $CD4^+$ Th1 cells induced during *T. gondii* infection were capable of simultaneously producing IL-10 as well as IFN-γ, and that this population of cells was important in limiting IL-12 production by APCs (Jankovic et al., 2007). Together, these findings indicate that regulation of the inflammatory response by IL-10 is critical for maintaining the balance of immunity vs. pathology.

Whereas IL-10 inhibits Th1 responses through its effects on APCs, transforming growth factor-β (TGF-β) has direct inhibitory effects on Th1 cells. TGF-β can also act to inhibit IL-12-induced pro-inflammatory cytokine production by NK cells (Hunter et al., 1995a). When TGF-β was neutralized following *T. gondii* infection, normally resistant mice suffered from acute inflammation and necrosis of the gut tissue (Buzoni-Gatel et al., 2001). Another more recently described cytokine, IL-27, has also been show to downregulate the inflammatory responses induced during toxoplasmosis. This cytokine is

specifically important in limiting host pathology induced by IFN-γ (Villarino et al., 2003) as well as IL-17 (Stumhofer et al., 2006). Overall, these studies illustrate some of the pathways that act to balance the immune system as it attempts to combat invading pathogens, especially in the context of chronic infection.

1.2 NF-κB in Immunity

The nuclear factor-κB (NF-κB) family is an evolutionarily conserved and ubiquitous family of transcription factors present in many species, from Drosophila to mice to humans. This section will introduce these transcription factors, focusing on their role in immune cell development and function. The Hunter laboratory has had a long-standing interest in how this family of transcription factors regulates innate and adaptive immunity to *T. gondii,* and for my thesis work I focused on the one subunit that is expressed only in immune cells, c-Rel, and how this transcription factor contributes to the development of protective $CD8^+$ T cell responses.

1.2.1 Introduction to NF-κB: Signaling

There are five different members in the mammalian NF-κB family of transcription factors, belonging to different classes, which are either synthesized as mature products (Rel A, Rel-B and c-Rel) or which must first undergo proteolytic processing (p105/p50 and p100/p52). Inactive NF-κB molecules are held in the cytoplasm through association with inhibitory members of the IκB family, which consists of 7 members (IκBα, IκBβ, IκBε, IκBγ, Bcl-3, and the p100 and p105 precursor proteins)

(Hayden et al., 2006). The IκB and NF-κB molecules interact via the Rel homology domain present on NF-κB proteins, which contains the nuclear localization sequence required for entry to the nucleus upon activation. NF-κB family members are expressed in every cell type and control numerous pathways involved in responses to infection, inflammation and injury (Gilmore, 2006). Because of the extensive list of factors able to induce NF-κB activation, signaling via this pathway is highly regulated and dysregulated function has been associated with abnormal cellular proliferation and lymphomas (Jost and Ruland, 2007). NF-κB signaling can occur via either a classical or a non-canonical pathway depending on the activating stimulus. Classical NF-κB signaling is mediated by the p50, c-Rel and RelA subunits, and is typically induced by T or B cell antigen receptor signaling, co-receptor activation, cytokines including TNF-α and IL-1, or TLRs (Figure 3). The non-canonical NF-κB signaling pathway uses the p52 and Rel-B subunits, and is activated by a specific set of stimuli that includes CD40L, BAFF (B cell Activating Factor) and Lymphotoxin-β.

Different NF-κB family members may serve overlapping roles in certain situations but non-redundant roles in others, complicating the study of their function. For instance, deletion of Rel A is embryonic lethal due to TNF hypersensitivity in the liver (Beg et al., 1995). p50 is also widely expressed, and its deletion leads to decreased generation of marginal zone as well as peritoneal B cells, as well as defective Th1 and Th2 responses (Artis et al., 2005; Cariappa et al., 2000; Grumont et al., 1998; Harris et al., 2010; Sha et al., 1995). Mice lacking Rel B are missing certain dendritic cell populations and are unable to form lymph node or Peyer's patch structures(Burkly et al.,

1995; Weih et al., 2001). Table 1 summarizes the susceptibility of different NF-κB knockout mice in a variety of infectious and inflammatory settings; these findings indicate that while some commonalities may be found between the 5 NF-κB family members, each can also play distinct roles.

1.2.2 c-Rel in immune cell development and function

Though different NF-κB family members have also been shown to be involved in a variety of cellular processes in diverse cell types including neurons, hepatocytes and osteoclasts, for the purposes of this thesis I will now focus on c-Rel. c-Rel was first identified as a proto-oncogene closely related to the avian Rel gene known to induce lymphomas in birds (Moore and Bose, 1988; Moore and Bose, 1989). Unlike other NF-κB family members, the expression of c-Rel is mainly limited to the immune system, in lymphoid and myeloid populations (Brownell et al., 1987; Simek and Rice, 1988). Early studies in c-Rel$^{-/-}$ mice indicated that although major immune cell populations could develop normally, lymphocytes showed major defects in antigen-induced activation, proliferation and effector function (Kontgen et al., 1995; Tumang et al., 1998). While c-Rel is required for antigen-driven proliferation of B cells, it does not appear to affect their lymphopenia-induced homeostatic expansion (Cabatingan et al., 2002), suggesting that cytokine-driven proliferation is regulated differently than mitogen-induced proliferation. c-Rel has also been demonstrated to play an important role in the development of marginal zone B cells (Cariappa et al., 2000), as well being required for isotype class switching (Harling-McNabb et al., 1999; Zelazowski et al., 1997). c-Rel is also associated with the inhibition of apoptosis, and accordingly the pro-survival molecule

Figure 3. Overview of NF-kB Signaling

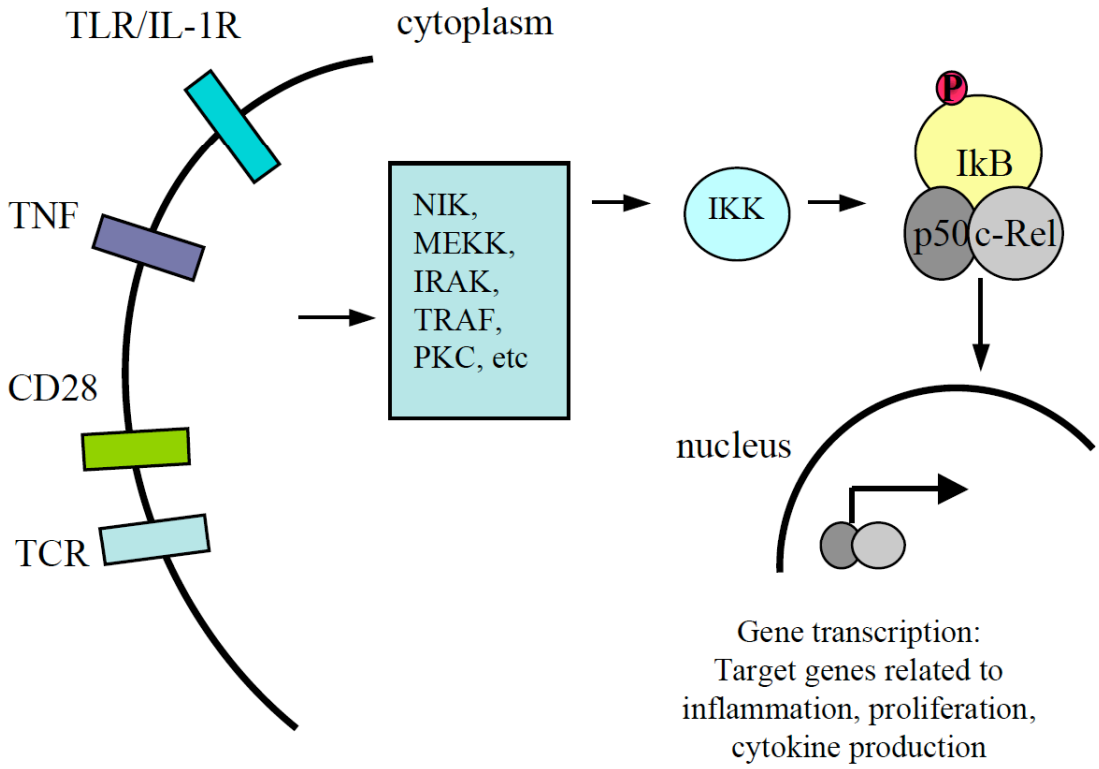


Figure 3. NF-kB is initiated through a number of different signaling pathways, including T cell and B cell receptors, co-stimulatory molecules like CD28, and cytokine receptors including TNF and IL-1. These pathways act through many different proteins, some of which are noted in the box above. Activation of IKK leads to degradation of inhibitory IkB molecules and release of NF-kB. Homo- and heterodimers of NF-kB proteins enter the nucleus upon activation, where they can bind to DNA and initiate target gene transcription.

Table 1. Phenotype of single gene NF-κB knockout mice: role in immune cells and during pathogen challenge. Unless otherwise noted, data refer to experiments conducted in single gene knockout mice.

Deficiency	**Functional abnormalities**	**Susceptibility to infection**
NF-κB1 (p50)	B cell proliferation and isotype switching;	Increased susceptibility to: *Leishmania major, Trichuris muris, Entamoeba histolytica*
NF-κB2 (p52)	Peyer's patch and germinal center formation;	Increased susceptibility to: *L. major, T. muris, T. gondii*
RelB	Germinal center formation and lymph node architecture; thymic atrophy; splenalomegaly	Increased susceptibility to: *T. gondii, Listeria monocytogenes, LCMV*
c-Rel	B and T cell proliferation and effector function; isotype switching	Increased susceptibility to: *T. gondii, L. major, L. monocytogenes* Not required for resistance to: *T. muris,* influenza virus
Rel-A (chimeras)	Macrophage nitric oxide	Increased susceptibility to: *L. major*

Bcl-x_L was shown to be a target of c-Rel in B cells (Owyang et al., 2001). In summary, these studies indicate a major role for c-Rel in the generation, survival and function of B lymphocytes. While biochemical studies have revealed direct binding by c-Rel to the promoters of a subset of genes in T cells, it is thought that the expression of other genes including IL-5, IFN-γ and TNF-α are affected indirectly. One potential mechanism that explains this outcome is the suboptimal production of IL-2 by c-Rel$^{-/-}$ CD4^{+} T lymphocytes, and the correspondingly poor proliferation of T cells in the absence of this growth factor. In T cells, however, this defective proliferation, as well as production of some cytokines, could be rescued with exogenous IL-2 (Gerondakis et al., 1996; Liou et al., 1999). Consistent with these early studies, IL-2 was later shown to be a c-Rel target gene that is activated following binding of c-Rel to the CD28 response element in T cells (Huang et al., 2001). Recent gain-of-function and loss-of-function experiments in CD4^{+} T cells found that none of the genes studied showed a complete dependence on c-Rel, rather its presence or absence influenced the expression levels of target genes depending on the context (e.g. cell type or activating stimulus) (Bunting et al., 2007).

c-Rel is also present in accessory cell compartments and several defects have been described. In macrophages, c-Rel has been shown to bind to the IL-12p40 promoter following LPS stimulation (Sanjabi et al., 2000). However in DC, c-Rel specifically induces IL-12p35 transcription, indicating that c-Rel has differential effects on cytokine production, depending on cell type (Grumont et al., 2001). Despite this defect in the ability of c-Rel$^{-/-}$ accessory cells to produce IL-12, mice infected with *T. gondii* could overcome this block and produce IL-12 independently of c-Rel (Mason et al., 2002). In

another study, bone marrow-derived DC were also found to be impaired in the production of IL-23 p19 (Carmody et al., 2007a), which is likely why c-Rel$^{-/-}$ mice are resistant to the development of colitis induced by *Helicobactor hepaticus* or by transfer of CD4^{+}CD45RB^{hi} T cells (Wang et al., 2008). In addition to regulating cytokine production in innate immune cells, c-Rel also influences the function of some APCs. A specialized subset of DC called plasmacytoid DC was reduced two-fold in c-Rel$^{-/-}$ mice, and these cells also had specific defects in production of IL-12p70 (O'Keeffe et al., 2005). Additionally, c-Rel$^{-/-}$ DCs had a defect in their ability to cross-present antigen in a model where mice were immunized with irradiated OVA-pulsed splenocytes (Mintern et al., 2002). The possibility that cross-presentation was impaired following immunization with *T. gondii* will be discussed further in Chapter 4.

In the context of infection studies with c-Rel$^{-/-}$ mice, the absence of this transcription factor contributes to increased susceptibility to some but not all pathogens. For instance, immunity to *T. muris* is not affected in c-Rel$^{-/-}$ mice, indicating that this transcription factor is not essential for the generation of Th2 immunity (Artis et al., 2002). Immunity to influenza infection also seems minimally affected by the loss of c-Rel because clearance of virus during acute infection, which is mediated by CD8^{+} T cells, was only slightly delayed (Harling-McNabb et al., 1999). In contrast, Th1-mediated immunity to other pathogens including *T. gondii* and *L. major* is severely compromised in c-Rel$^{-/-}$ mice (Grigoriadis et al., 1996; Mason et al., 2004b). However, loss of c-Rel can also lead to resistance against Th1-mediated immune pathology. When c-Rel$^{-/-}$ mice received H-2 mismatched islet allografts, they had increased survival of transplanted

tissue in comparison to WT mice, associated with decreased infiltration of the graft and depressed T cell responses (Yang et al., 2002). c-Rel$^{-/-}$ mice are also resistant to the development of diabetes (Lamhamedi-Cherradi et al., 2003). In humans, over-expression of c-Rel has been linked with malignancies (Houldsworth et al., 1996; Lu et al., 1991), systemic lupus erythematosus (Burgos et al., 2000) and more recently with rheumatoid arthritis (Dieguez-Gonzalez et al., 2009; Eyre et al., 2009; Gregersen et al., 2009). While clearly the role of c-Rel during a coordinated immune response is complex, a model illustrating immunity to *T. gondii* and the aspects during this response where c-Rel is implicated is shown in Figure 4.

In the following chapters, my thesis work looking at CD8$^+$ T cell responses to *T. gondii* will be presented. While much is known about the regulation of CD8$^+$ T cell responses to infection in different models, unanswered questions remained in areas related to the influence of the inflammatory environment on the development of T cell responses, and in the development and maintenance of memory during parasite infection. This introduction was meant to highlight the complex biology of *T. gondii* infection, and also the complicated nature of the immune response and some of the many factors that regulate the responses of different immune cell subsets. My thesis work will demonstrate the development of adaptive immunity to a replication-deficient parasite strain and discuss the role of the inflammatory environment, specifically the key contributions of the cytokine IL-12. Additionally, I will focus on the NF-κB family member c-Rel and its role in supporting CD8$^+$ T cell development, and hypothesize about its role in T cell memory.

Figure 4. Model for the role of c-Rel during the immune response to *T. gondii*

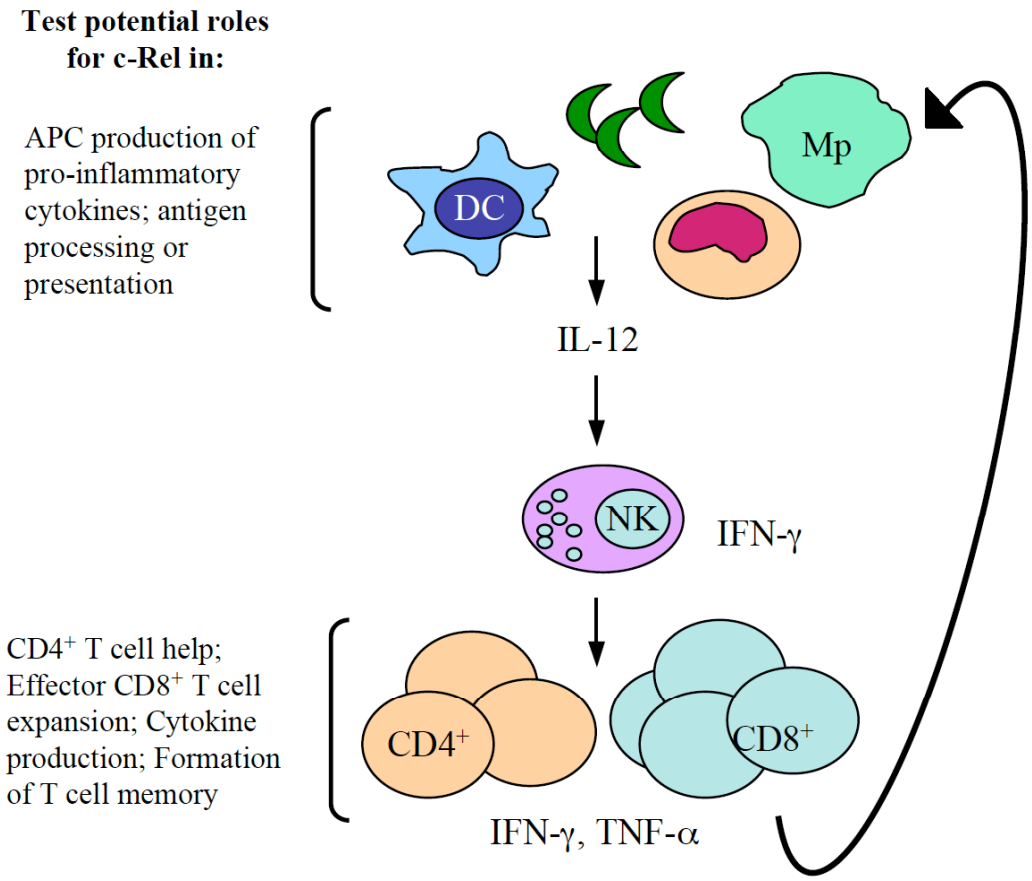


Figure 4. c-Rel could play a role in innate or adaptive immune responses; its potential roles in each compartment during the immune response to *T. gondii* are hypothesized above. Of particular interest is the role of c-Rel in the inflammatory environment, in the expansion of $CD8^+$ effector T cells, and in the maintenance of $CD8^+$ T cell memory.

Chapter 2 : Materials and Methods

Mice

C57BL/6, Thy1.1, C57BL/6-*Prf1*^{tm1Sdz}/J (Kagi et al., 1994) and B6.MRL-*Fas*^{lpr}/J mice (Andrews et al., 1978) were purchased from Jackson Laboratory (Bar Harbor, Maine). In some experiments C57BL/6 and Thy1.1 mice were purchased from NCI (Frederick, MD). B6.SJL-*Ptprc*^{a}/BoyAiTac (CD45.1) mice were purchased from Taconic. c-Rel$^{-/-}$ mice were originally obtained from H.C. Liou (Weill Medical College of Cornell University, New York, NY) (Tumang et al., 1998) and were bred at the University of Pennsylvania. B6[TG]TCR-OT-1 RAG1[KO] (Hogquist et al., 1994; Mombaerts et al., 1992) were obtained through the NIAID Exchange Program (Taconic line 4175). DPE-GFP transgenic mice, which express GFP in all T cells, were originally obtained from U.H. von Andrian (CBR, Harvard, Boston MA) and were crossed to OT1 TCR transgenic mice, resulting in OT1 transgenic TCR that express GFP (OT1-GFP) (Mrass et al., 2006). All mice were maintained under specific pathogen-free conditions and all animal work was done in accordance with the Institutional Animal Care and Use Guidelines of the University of Pennsylvania.

Parasites and Infections

Tachyzoites were grown in human foreskin fibroblast monolayers in Dulbecco modified Eagle medium containing either 1% FCS (CPS and RH parasites) or 10% FCS (Pru parasites), and 1% penicillin-streptomycin. CPS parasites were cultured in media

containing 0.2 mM uracil, and ovalbumin-transgenic parasites were maintained in media additionally containing 20 μM chloramphenicol. For all immunization experiments using CPS-OVA, mice were injected intraperitoneally (i.p.) with 10^5 parasites. For challenge experiments, mice were given 10^3 RH-OVA tachyzoites (RH parental strain expressing secreted ovalbumin) or 10^6 CPS-OVA. For all adoptive transfer experiments, mice received 10^5 Pru-OVA tachyzoites i.p.

T cell Depletions and Transfers

For T cell depletion experiments, mice received two doses of 0.5 mg of anti-CD4 (clone RM4.5) or anti-CD8 (clone 2.43) in the week prior to challenge. For depletion of IL-12, mice received two injections of anti-IL12p40 (clone C17.8) on day -1 and day 2 of immunization. For rescue of antigen-specific responses, c-Rel$^{-/-}$ mice received 3 intraperitoneal (i.p.) injections containing 500 ng of recombinant IL-12p70 (eBioscience) on days 0, 1 and 2 of infection. For adoptive transfer experiments, splenic and lymph node T cells were isolated via negative selection (R&D Systems, Minneapolis, MN); mice received 5 x 10^5 WT or c- Rel$^{-/-}$ OT1 cells that were transferred into recipient mice intravenously via retro-orbital injection 24 hours prior to challenge. For assessment of memory responses, T cells were isolated 30 days following immunization and 10^7 immune cells were transferred intravenously one day prior to secondary challenge. Mice were anesthetized with isoflurane prior to intravenous injection.

***In vitro* T cell responses**

Mice were sacrificed by CO_2 asphyxiation. Spleen, lymph node (LN) and peritoneal exudate cells (PECs) were harvested and dissociated into single cell suspensions in complete RPMI 1640 (Gibco/Invitrogen, Carlsbad, CA) containing 10% heat-inactivated FCS, 10 U/ml penicillin, 100 μg/ml streptomycin, 2mM glutamine, 25 mM HEPES, 0.1 mM non-essential amino acids and 50 μM 2-beta mercaptoethanol (2ME). For analysis of peripheral blood mononuclear cells, blood was collected and mixed with sodium citrate prior to lysis of erythrocytes with 0.86% ammonium chloride buffer. Spleen and LN cells (5×10^5/well) and PECs (10^5/well) were plated out in triplicate in 96-well round bottom plates (Costar, Carlsbad, CA) and cultured to assess for cytokine production. Cells were restimulated with anti-CD3 (1 μg/ml), soluble *T. gondii* antigen (STAg; 10-25 μg/ml), 500 μg/ml OVA (Worthington Biochemical Corporation, Lakewood, NJ) or 1μg/ml OVA peptide (SIINFEKL) (CHI Scientific, Maynard, MA). Supernatants were removed after approximately 48 hours and assayed for the production of IFN-γ by enzyme-linked immunosorbent assay (ELISA) as previously described.

Acute IL-12 Responses

For assessment of acute IL-12 production, WT and c-Rel$^{-/-}$ mice were infected as noted above. At 72 hours following challenge, whole blood was collected and incubated at room temperature prior to centrifugation for 10 min at 14,000 RPM to facilitate serum isolation. Serum was assayed for IL-12p70 as well as IL-12p40 by ELISA. PECs were collected and plated out at 10^6 cells/well, and incubated for 24 hours in the absence of additional antigen stimulation. IL-12 levels were then assessed by ELISA.

B3Z Antigen Presentation assay

Splenic dendritic cells (DC) were isolated from WT and c-Rel$^{-/-}$ mice, at 4 days post-infection with 10^4 Pru-OVA parasites. Cells were sorted by staining the cell suspension with CD11c microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany), and selection of the DC subset occurred by running the cell suspension through an LS column (Miltenyi). The B3Z cells have been described previously (Karttunen et al., 1992). This hybridoma cell line expresses a β-galactosidase reporter that becomes active following TCR stimulation, and its TCR is specific for ovalbumin. Titrated numbers of DC from naïve or infected WT and c-Rel$^{-/-}$ mice were plated out in medium without phenol red. B3Z cells were added in the absence of exogenous antigen. β-galactosidase activity was detected by adding chlorophenol red-β-D-galactopyranoside (CPRG; Calbiochem, San Diego, CA) at a final concentration of 100 μM, and absorbance was measured at 562 nm. Activation was expressed as increase in absorbance, relative to values obtained in the absence of antigen, as described previously (Dzierszinski et al., 2007).

Flow cytometry

For identification of OVA-specific T cell responses, single-cell suspensions were washed in FACS buffer (PBS, 2 mM EDTA, 2% BSA) and incubated for 15 minutes with Fc block (FACS Buffer containing 1 μg/ml 2.4G2 (BD Pharmingen, San Jose, CA) and 1 μg/ml rat and mouse IgG (Caltag)). Cells were stained with MHC class I/tetramer complexes of H-2K^b/SIINFEKL conjugated to phycoerythrin (PE) or allophycoerythrin (APC) (Beckman Coulter Immunomics, or generous gift from John Wherry (Wistar Institute, Philadelphia PA)) for 25 minutes at room temperature. The cells were then

washed and stained for other surface markers for 15 minutes at 4° C. Naïve mice were used as controls to assess levels of background staining obtained with the tetramer. The following monoclonal antibodies were used for phenotypic staining: CD8 (conjugated to fluorescein (FITC,) Peridinin Chlorophyll Protein Complex (PerCP) or APC), CD127-biotin, CD122-biotin, CD62L-APC or APC Alexa fluor 700 (BD Biosciences); TNF-α FITC, IFN-γ-APC, IL-2 PE, KLRG1-APC, Streptavidin-APC (eBioscience, San Diego, CA); anti-human Granzyme B-APC (Caltag, Carlsbad, CA).

For intracellular cytokine analysis, splenocytes or PECs were incubated (cell and antigen concentration as noted above) for 5.5 hours total, with the addition of 10μg/ml Brefeldin A (Sigma) for the final 4 hours. Cells were first stained for surface markers, followed by fixation overnight with 2% PFA (Electron Microscopy Sciences, Hatfield, PA). Cells were permeabilized with 0.1% saponin and then stained for intracellular cytokines for 1 hour at 4°C. Flow cytometry samples were collected on a FACSCalibur or FACSCanto machine (BD) and analyzed with FlowJo software (Tree Star Inc. Ashland, OR).

***In vivo* CTL assay**

Cytolytic activity was assessed using the *in vivo* CTL assay, modified slightly from previous protocols (Barber et al., 2003). Briefly, spleen and LN cells from CD45.1 mice were pooled and pulsed with 1 μg/ml OVA peptide (CHI Scientific) for 1hour at 37°C. Cells were washed extensively in PBS, labeled with CFSE (Molecular Probes/Invitrogen) at a concentration of 5μM (cells pulsed with OVA peptide) or 0.1μM Carboxyfluorescein succinimidyl ester (CFSE) (unpulsed cells). Cells were then counted and resuspended at a

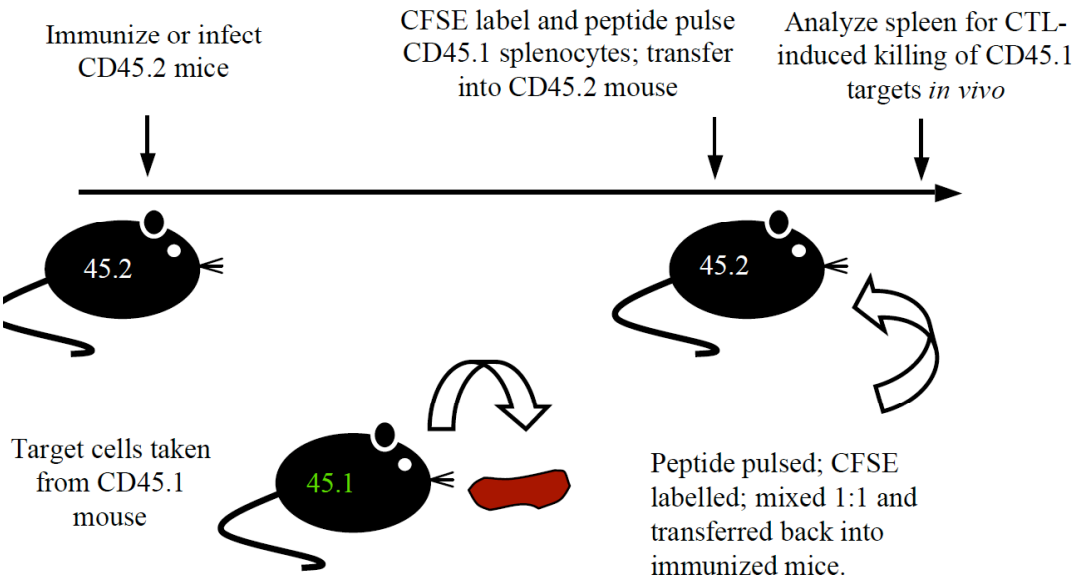
1:1 ratio. $6x10^6$ total cells ($3x10^6$ peptide-pulsed and $3x10^6$ unpulsed) were then transferred intravenously to anesthetized recipient mice. Mice were sacrificed 4-24 hours later and spleens were analyzed for specific lysis of the peptide-pulsed population by flow cytometry by gating on $CD45.1^+$ donor cells. Specific lysis was calculated as described previously (Barber et al., 2003). A diagram of the lysis assay and calculation of lysis activity is shown below in Figure 5.

Statistics

Statistical analyses were completed with Prism (Graphpad Software, La Jolla, CA) using Student's t test, or one-way ANOVA where indicated. Where appropriate, data are shown as mean ± standard deviation. The Kaplan-Meier test was used to measure significant differences between survival curves of naïve versus immunized c-$Rel^{-/-}$ mice, and unmanipulated versus T cell-depleted immune mice following RH-OVA challenge.

Figure 5. Outline of *in vivo* cytolysis assay

A



B

$$\text{Percent killing} = 100 - 100^{*} \left\{ \left(\frac{\%\ \text{peptide infected}}{\%\ \text{unpulsed infected}} \right) \div \left(\frac{\%\ \text{peptide uninfected}}{\%\ \text{unpulsed uninfected}} \right) \right\}$$

Figure 5. Model slide for assessment of *in vivo* cytotoxic activity. (A) CD45.2 mice are immunized. Spleens are removed from CD45.1 mice, and half are pulsed with 1uM OVA peptide. Cells are labeled with two concentrations of CFSE; OTI-peptide pulsed target cells are CFSE high (2.5uM) and unpulsed cells are CFSE low (50nM), mixed 1:1 and transferred into immunized mice intravenously. Target cells are left for 4- 16 hours, and then recipient mice are analyzed for loss of OVA-pulsed cells. (B) Formula to calculate specific cytotoxicity based on values determined from naïve mice, as measured by flow cytometry.

Chapter 3: The CPS-OVA Model System

3.1 Introduction to the CPS-OVA system

While much has been learned about the factors that lead to protective immunity to *T. gondii*, relatively little is known about what is required for optimal generation of antigen-specific T cell responses. To better define some of the factors that lead to protective immunity and the development of antigen-specific $CD8^+$ T cell memory, a system had to be developed to enable long-term tracking of infection-induced populations. Therefore, in collaboration with colleagues in the Department of Biology, Type I (RH) and Type II (Pru) strains of *T. gondii* that secreted the model antigen ovalbumin (OVA) were generated (Dzierszinski et al., 2007; Pepper et al., 2004). The Pru-OVA parasite was used in studies by the Hunter laboratory to show the activation and expansion of adoptively transferred transgenic $CD4^+$ T cells. It was soon realized that endogenous $CD8^+$ T cell responses were also induced by parasite secretion of the model antigen OVA, and that these responses could be tracked using MHC Class I/OVA tetramers.

The aim of the work presented in this chapter was to create better understanding of how the adaptive immune response to *T. gondii* is generated, which will be helpful for the development of an effective vaccine. Various *T. gondii* protein or DNA vaccines have been tested in mice and have provided varying levels of protection (Scorza et al., 2003; Yap et al., 1998). Perhaps the most effective protection against *T. gondii* has been provided by infection with an attenuated temperature-sensitive strain of parasite called ts-

4; however, this strain possesses the ability to replicate and cause disease in immuno-deficient as well as immunocompetent hosts and so could not be used in humans (Sayles and Johnson, 1996). A possible solution to this safety issue is to genetically modify parasites so they are unable to replicate *in vivo*, and would be incapable of reverting to a virulent phenotype. One such clone was generated by knocking out a regulatory enzyme of the *de novo* pyrimidine biosynthesis pathway, carbamoyl phosphate synthetase (cps)-II (Fox and Bzik, 2002), a mutation that renders the parasites unable to replicate in the absence of exogenous uracil. The cps-II strain is therefore non-virulent in mice; even mice that lack IFN-γ survive infection with this organism (Fox and Bzik, 2002). Further, immunization with cps-II protects WT mice following challenge with the virulent RH strain (Fox and Bzik, 2002). At the time these studies were initiated, however, little was known about the nature of the adaptive immune response that these parasites induced. Consequently, to allow the tracking of an endogenous $CD8^+$ T cell population, the cps-II parasites were engineered to express a secreted form of the model antigen ovalbumin (hereafter referred to as CPS-OVA).

The experiments presented here examine the kinetics, phenotype and function of endogenous antigen-specific $CD8^+$ T cells generated in response to the CPS-OVA parasites. Immunization-induced protection required T cells at the time of challenge, and the $CD8^+$ T cell subset was most protective. The peak of $CD8^+$ T cell expansion to CPS-OVA was seen at day 10 in both the site of infection as well as secondary lymphoid tissues. Unexpectedly, despite being replication-deficient, these parasites induced an OVA-specific response that was comparable in magnitude and more rapid than that observed using a replicating parasite, Pru-OVA. Optimal generation of the antigen-

specific cells in response to CPS-OVA required the presence of $CD4^+$ T cells at the time of immunization. Nonetheless, the $CD8^+$ T cells that were generated in the absence of $CD4^+$ T cell help were able to produce IFN-γ and were cytolytic. $CD4^+$ T cell help also was critical for the upregulation of the killer-like lectin receptor G1 (KLRG1) on $CD8^+$ T cells, a marker upregulated by effector T cells in a number of infection models (Blaser et al., 1998; Joshi et al., 2007; Robbins et al., 2003). Cytolytic activity was mediated mainly via a perforin-dependent pathway while Fas-FasL interactions were not required. This work provides some of the first data regarding the phenotype and function of antigen-specific cells induced by a non-replicating strain of *T. gondii,* and suggests that an effective vaccine targeting cell-mediated immunity should engage both $CD4^+$ and $CD8^+$ T cell subsets.

3.2 T cells are required for CPS-OVA induced protective immunity

Earlier work demonstrated that cps1-1 parasites could provide protective immunity to challenge with the virulent RH strain of *T. gondii* (Fox and Bzik, 2002). However, these studies did not provide an analysis of the adaptive immune response to immunization, or indicate which cell types were important for protection. In order to define which T cell subsets were required for CPS-induced protective immunity, mice were injected intraperitoneally with a single dose of 10^5 CPS-OVA parasites, and 30 days

Figure 6. CPS-OVA immunization protects mice from virulent RH-OVA challenge

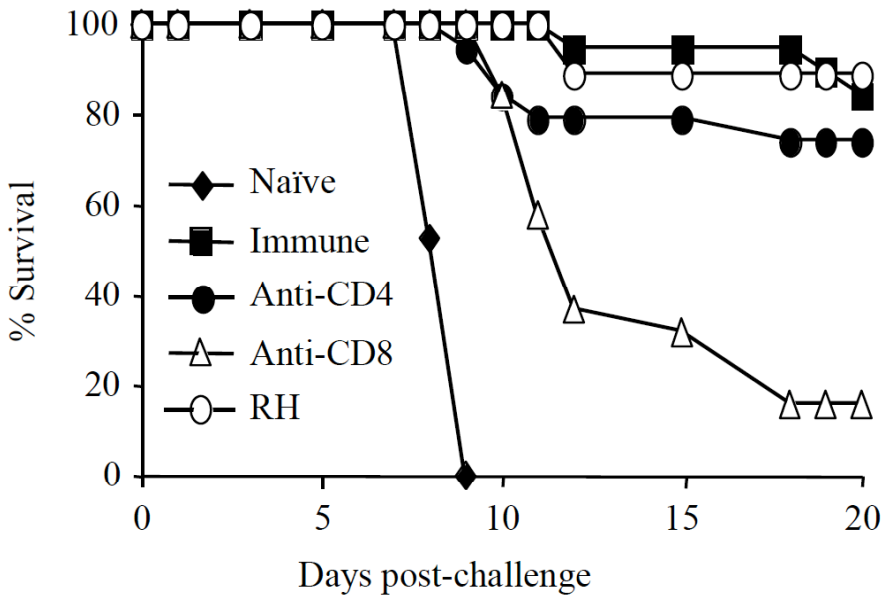


Figure 6. Immunization protects mice from RH challenge in a T cell-dependent manner. Mice were immunized once i.p. with 10^5 CPS-OVA tachyzoites and 30 days later were challenged i.p. with 10^3 RH-OVA tachyzoites. For depletion experiments, immunized mice were depleted of $CD4^+$ or $CD8^+$ T cells 1 week prior to challenge. Survival was monitored daily. Combined results from 2 experiments with a total of 10 mice per group.

Adapted from KA Jordan et al, Infection and Immunity 2009.

Figure 7. Induction of OVA-specific $CD8^+$ T cell responses

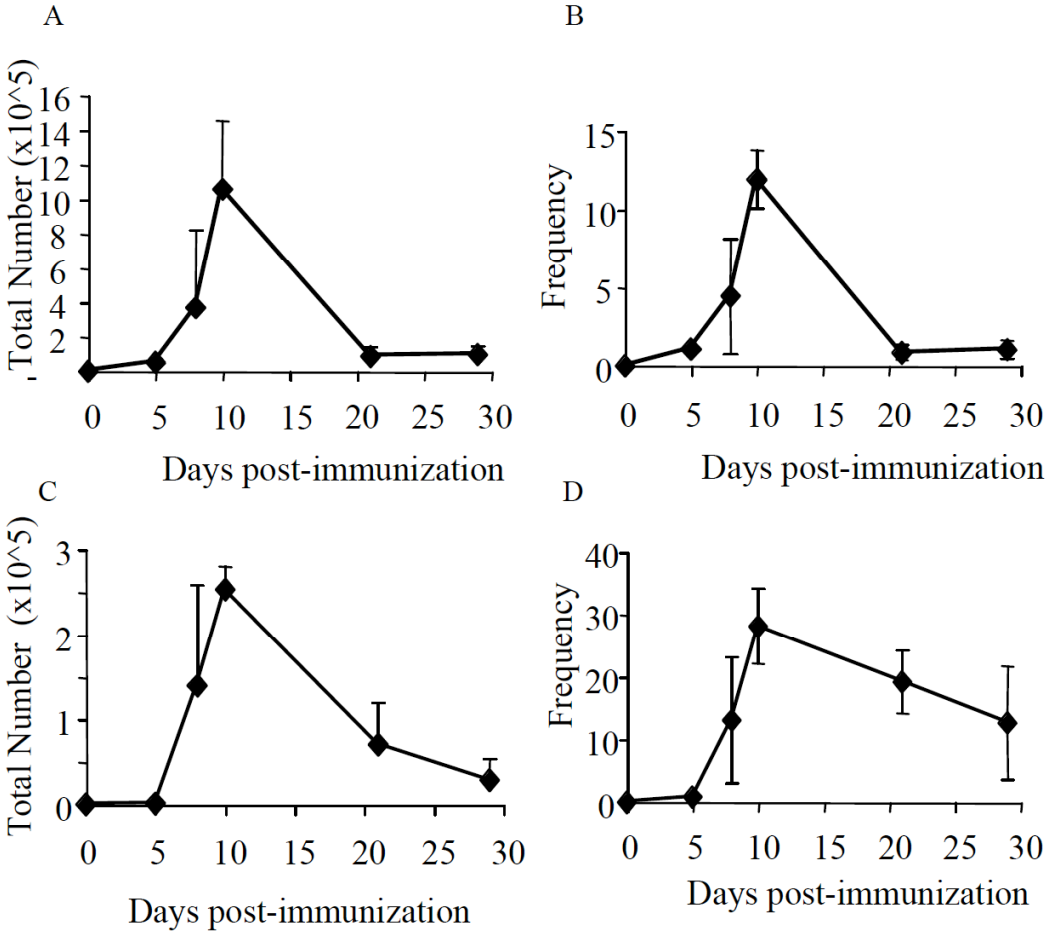


Figure 7. Mice were immunized once i.p. with 10^5 CPS-OVA tachyzoites and OVA-specific responses were measured over time. (A) Total number of OVA-specific cells in the spleen. (B) Frequency of OVA-specific cells in the spleen (out of $CD3^+CD8^+$ lymphocytes). (C) Total number of OVA-specific cells in the PECs. (D) Frequency of OVA-specific cells in the PECs (out of $CD3^+CD8^+$ lymphocytes). Representative data from 1 of 2 similar experiments with at least 3 mice per time-point.

Adapted from KA Jordan et al, Infection and Immunity 2009.

later were challenged with the virulent Type I strain RH-OVA (or the parental strain lacking expression of OVA, RH). Unimmunized mice succumbed to RH-OVA infection by day 12, while mice immunized with CPS-OVA survived this challenge (Figure 6). To clarify whether or not T cells were important for this protection, immunized mice were depleted of either $CD4^+$ or $CD8^+$ T cells one week prior to RH-OVA challenge. Depletion of $CD8^+$ T cells resulted in increased susceptibility to RH-OVA challenge ($p = 0.0032$ by one-way ANOVA), while $CD4^+$ depletion had only a minor effect on survival (Figure 6). Thus, in this model of immunization with a single dose of 10^5 CPS-OVA parasites, while both $CD4^+$ and $CD8^+$ T cells contributed to resistance to challenge, $CD8^+$ T cells provided the most protection. As shown in Figure 6, CPS-OVA immunization was also able to provide resistance to challenge with the parental strain (RH), indicating that CPS-OVA induces a polyclonal response that is not completely targeted toward OVA.

3.3 Immunization with a replication-deficient parasite induces a robust endogenous $CD8^+$ T cell response and upregulation of activation markers

Because the parasite used in these experiments expressed the model antigen OVA, studies were performed to determine if we could track the endogenous $CD8^+$ T cell response using an OVA-specific MHC Class I tetramer. These experiments revealed that OVA-specific cells could be readily detected as early as 5 days following immunization, though as a proportion of the $CD8^+$ T cell population the frequency of OVA-specific $CD8^+$ T cells was much higher in the PECs (Figure 7D) compared to the spleen (Figure 7B). Kinetic analysis indicated that the peak of this response occurred 10 days post-infection, after which point it declined, though low numbers of OVA-specific cells were

maintained at least 30 days in these sites (Figure 7A, C). Though the magnitude of OVA-specific $CD8^+$ T cells peaked at day 10 in both sites, the antigen-specific population contracted more slowly in the PECs than in the spleen.

The use of tetramers to identify the OVA-specific $CD8^+$ T cell population allowed us to analyze the expression of phenotypic markers including CD62L, KLRG1, CD122 and CD127 on parasite-specific cells. We found that at day 7, the majority of $tetramer^+$ cells in the spleen had upregulated KLRG1 and had downregulated CD62L (Figure 8, top panel), thus exhibiting an effector phenotype similar to what has been noted in other viral and bacterial models (Huster et al., 2004; Joshi et al., 2007; Kaech et al., 2003; Ku et al., 2000; Pearce and Shen, 2007). The $tetramer^+$ cells in the PECs were phenotypically similar to what was seen in the spleen (data not shown). Antigen-specific cells also showed higher expression of the IL-2/IL-15 receptor-β (CD122) compared to $tetramer^-$ cells. Consistent with recent publications using *Listeria monocytogenes* infection (Huster et al., 2004), expression of the IL-7Rα was decreased on $tetramer^+$ cells 7 days following immunization when compared to naïve $CD8^+$ T cells (Figure 8, shaded gray histogram). IL-7Rα was also downregulated on a small population of $tetramer^-$ $CD8^+$ T cells, which would include primarily naïve cells but also a small population of activated cells (data not shown).

Figure 8. Phenotype of OVA-specific effector and memory CD8$^+$ T cell responses

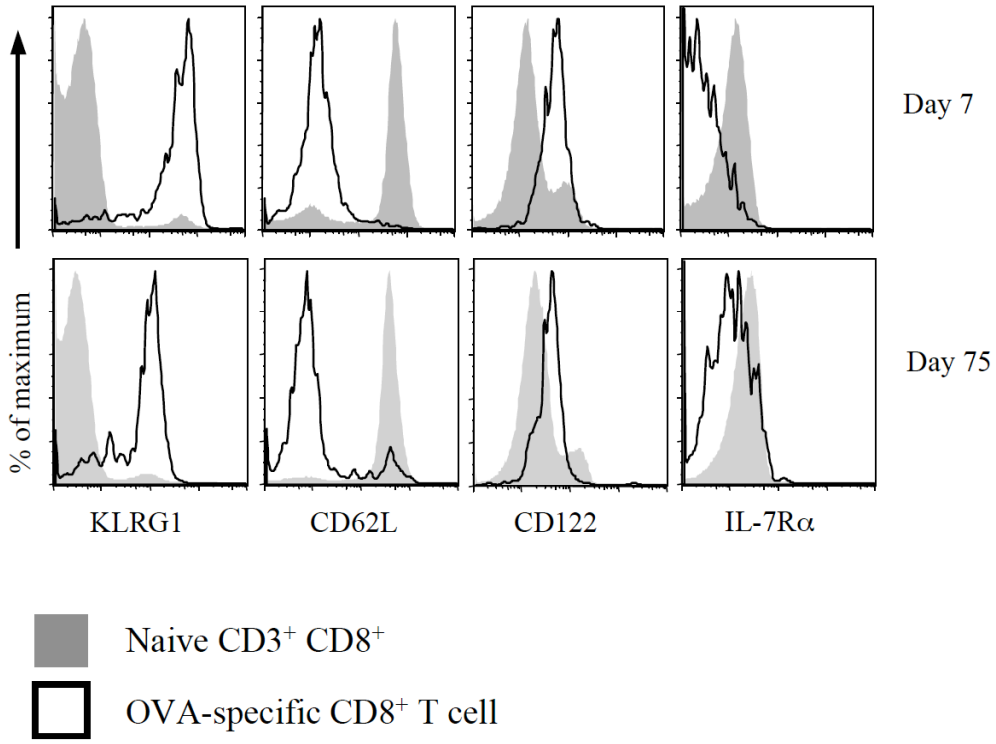


Figure 8. Mice were immunized once i.p. with 10^5 CPS-OVA tachyzoites and cells from the spleen were analyzed phenotypically by flow cytometry at the indicated times. Representative histograms from one mouse, gated on CD3$^+$CD8$^+$ T cells, with OVA-specific cells in the open histogram and naïve CD8$^+$ T cells shown in the gray histogram. Top row shows staining at day 7 post-immunization, and bottom row shows staining from day 75. These are representative data and each time-point was repeated at least 3 times.

Adapted from KA Jordan et al, Infection and Immunity 2009.

To assess the phenotype of the memory cell population, later time-points were also examined. Live parasites cannot be recovered from the PECs beyond 5 days after immunization (CD Dupont, personal communication), and it is difficult to consistently detect parasite DNA even as early as 10 days following immunization, so it is very unlikely that viable parasites persist in immunized mice for extended periods of time. As late as 75 days following immunization, the tetramer$^+$ cells for the most part maintained an effector-like KLRG1$^+$CD62L$^-$ phenotype (Figure 8, bottom panel). Expression of the high-affinity IL-7 receptor, which has been identified as a cytokine receptor expressed on long-term memory cells (Huster et al., 2004), was also examined. A representative example is shown in Figure 8, and analysis of the tetramer$^+$ population revealed that a proportion of OVA-specific cells expressed the IL-7Rα (67.7 ± 2.9%, n = 7 combined from 3 different experiments). Overall, these experiments demonstrated that antigen-specific cells at the site of infection as well as in secondary lymphoid tissues resembled an effector population up to 75 days following immunization.

As data emerged from these studies, it became apparent that the kinetics of the CD8$^+$ T cell response to CPS-OVA differs from what has been reported for replication-sufficient strains of *T. gondii*. While the antigen-specific response induced by CPS-OVA peaks approximately 10 days following immunization in both the spleen and PECs, work from other groups has demonstrated that optimal CD8$^+$ T cell responses induced by replicating parasites are not detectable until 2 weeks following infection (Kwok et al., 2003; Lutjen et al., 2006). To directly compare the development of antigen-specific responses, mice were given the same dose of 10^5 CPS-OVA or Pru-OVA tachyzoites intraperitoneally, and tetramer responses were assessed at two time-points. Pru-OVA and

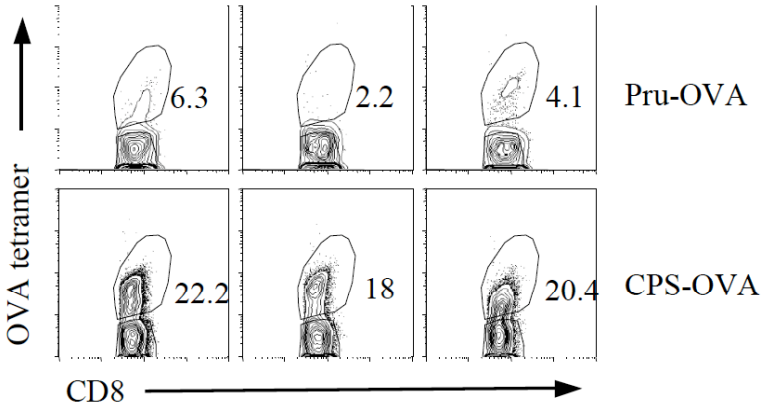
CPS-OVA (derived from an RH strain) are derived from different strains of *T. gondii* that have previously been shown to differ in virulence to the host (Saeij et al., 2006). In this comparison, CPS-OVA induced significantly higher frequencies of OVA-specific $CD8^+$ T cells in the spleen (Figure 9A) as well as in the PECs (data not shown) 8 days following infection. However, by day 14, the pattern had reversed such that Pru-OVA responses were now significantly higher in the spleen with a trend towards higher responses in the PECs, while CPS-OVA-induced $CD8^+$ T cell responses had started to contract (Figure 9B). However, work from our laboratory (Tait ED et al., submitted) has compared replicating and non-replicating Type 1 strains and demonstrates that CPS-OVA induces higher frequencies of antigen-specific $CD8^+$ T cells compared to its replication-sufficient parental strain, RH-OVA. Nevertheless, both CPS-OVA and Pru-OVA generate antigen-specific T cells with a similar effector-like phenotype at these time points, and OVA-specific cells express low levels of CD62L and CD127, and high levels of KLRG1 and CD122 (Figure 9C), as well as high levels of CD44 (data not shown).

3.4 OVA-specific $CD8^+$ T cells are cytotoxic

In addition to production of cytokines, another important function of antigen-specific $CD8^+$ T cells is the lysis of infected cells. Indeed, one week following immunization, many $tetramer^+$ splenocytes had upregulated expression of granzyme B, consistent with possible cytolytic activity (Figure 10A). While a proportion of the total

Figure 9. Comparison of CPS vs. Pru CD8$^+$ T cell responses

A



B

	Spleen day 8	PECs day 8	Spleen day 14	PECs day 14
Pru-OVA	0.81 ± 0.37	4.2 ± 1.22	6.76 ± 1.32	17.47 ± 2.85
CPS-OVA	6.69 ± 1.45	20.2 ± 1.2	1.55 ± 0.25	6.70 ± 0.37

C

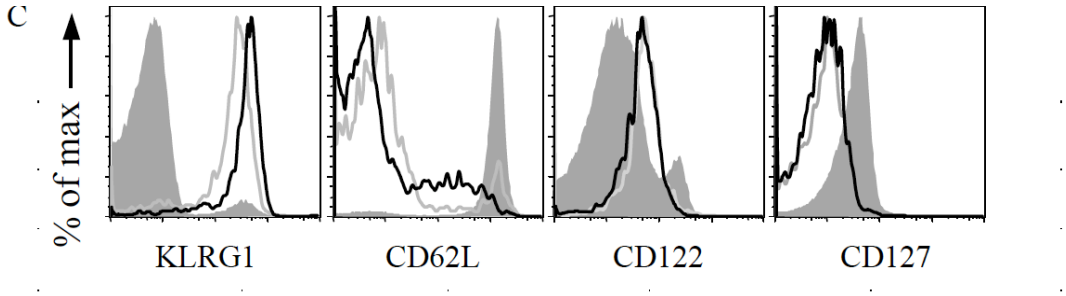


Figure 9. CPS-OVA induces antigen-specific CD8$^+$ T cells more rapidly than Pru-OVA. (A) Tetramer responses from the spleens of 3 individual mice 8 days following infection with 10^5 parasites of the indicated strain. Numbers represent frequency of tetramer$^+$ cells out of CD3$^+$CD8$^+$ cells. (B) Frequency of tetramer$^+$ cells in the spleen and PECs at day 8 and day 14, values represent average of 3 mice ± standard deviation. Significant difference of $p < 0.02$ (Pru-OVA vs. CPS-OVA) for all values except PECs day 14. Experiment was repeated twice with similar results. (C) Phenotype of naïve CD8$^+$ T cells (shaded gray), Pru-OVA tetramer$^+$ cells (black line) and CPS-OVA tetramer$^+$ cells (gray line).

Adapted from KA Jordan et al, Infection and Immunity 2009.

Figure 10. CPS-OVA immunization induces CD8$^+$ T cell cytolytic activity

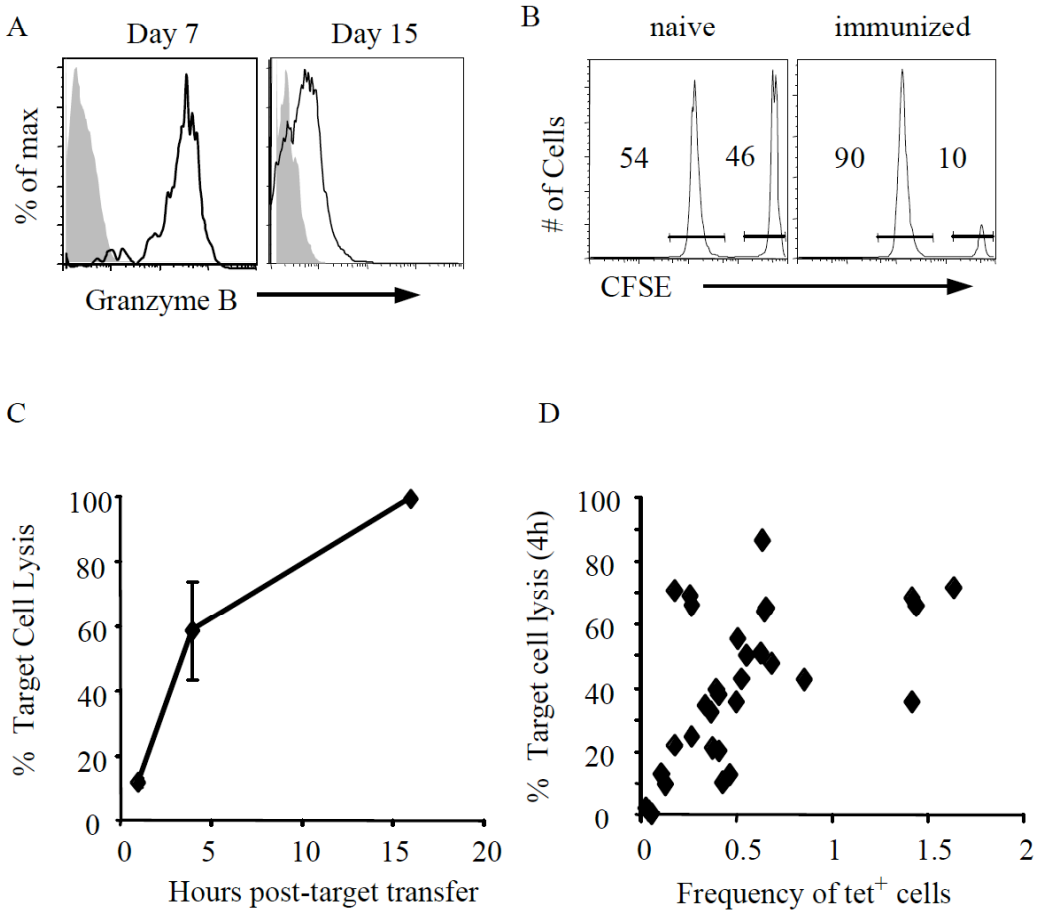


Figure 10. CPS-OVA immunization induces CD8$^+$ T cell cytolytic activity. (A) Expression of granzyme B over time (filled: naïve; black line: tetramer$^+$). (B) Mice were immunized i.p. with 10^5 CPS-OVA and given CFSE-labeled target cells; numbers represent the proportion of donor cells that are unpulsed (CFSE low) or OVA peptide-pulsed (CFSE high). (C) Target cell lysis of donor cells in the spleen over time. (D) Target cell lysis 4 hours following transfer is correlated with frequency of tetramer$^+$ cells.

Adapted from KA Jordan et al, Infection and Immunity 2009.

$CD8^+$ T cell population had also upregulated granzyme B, this frequency was lower when compared to the $tetramer^+$ population (5.5±1.6% vs. 70.3±8%). Two weeks following immunization, a decreased frequency (40.9±3.7%) of antigen-specific cells stained positive for this cytolytic effector molecule directly *ex vivo*, compared to what was seen at day 7. To directly assess the cytolytic ability of these cells, an *in vivo* CTL assay was developed based on earlier work (Barber et al., 2003). Briefly, unpulsed or OVA-peptide pulsed splenocytes from congenic CD45.1 mice were CFSE labeled, mixed at a 1:1 ratio, and injected into immunized (CD45.2) mice (as outlined in Figure 5). When immunized recipient mice were analyzed, loss of the OVA peptide-pulsed $CFSE^{hi}$ population indicated that CPS-OVA induced robust cytolytic activity in $CD8^+$ T cells. While cytolytic activity could be detected as soon as 4 hours after target cell transfer in the recipient spleens, maximum lysis was achieved after a 16-hour incubation of the target cells in the recipient mice (Figure 10B-C). Multiple experiments were combined from various time-points from 7 to 30 days following immunization and demonstrated a correlation between the level of cytotoxic activity and the frequency of antigen-specific cells present at the time of the CTL assay (Figure 10D).

To better understand the requirements for cytotoxic activity, WT and perforin-deficient mice were immunized with a single dose of 10^5 CPS-OVA. Studies with *L. monocytogenes* in perforin-deficient mice demonstrated that these animals generated increased frequencies of antigen-specific $CD8^+$ T cells (Badovinac et al., 2000), and this was proposed to be due to decreased killing of DC leading to increased antigen exposure following infection. Similar to what was seen in this model, $perforin^{-/-}$ mice immunized

with CPS-OVA generated an increased frequency of tetramer$^+$ cells (Figure 11A; p = 0.0004 by student's t test). Despite this, perforin$^{-/-}$ mice demonstrated significantly lower levels of cytolytic activity against peptide-pulsed target cells when analyzed either 4 (92.3±0.1% vs. 43.5±4.6%, p = 0.0005) or 16 (99.9±0.1% vs. 82.3±0.8%, p < 0.0001) hours following target cell transfer (Figure 11B); however, there was still a large perforin-independent component. In addition to granule-mediated cytotoxicity, CD8$^+$ T cells can also mediate cytolysis via Fas-FasL interactions. To examine the contribution of Fas to the perforin-independent pathway of cytolysis, Fas KO peptide-pulsed target cells were mixed with WT target cells and unpulsed cells and transferred into immunized mice. WT and Fas KO target cells were killed equally well, indicating that the perforin-independent cytotoxic activity generated in response to immunization is likely not mediated via the Fas-FasL pathway (Figure 11C). Because the kinetics of cytotoxicity induced by another killing mechanism, TNF-α, are much slower than the activity of perforin and granzyme (Ratner and Clark, 1993), it is unlikely that TNF-α-mediated killing was important during these assays.

3.5 Depletion of CD4$^+$ T cells abrogates effector CD8$^+$ T cell responses to CPS-OVA

Different models of infection have shown diverse requirements for CD4$^+$ T cell help in the generation of effector CD8$^+$ T cells. In the primary response to herpes simplex virus (Jennings et al., 1991) or *Mycobacterium tuberculosis* (Serbina et al., 2001), CD4$^+$ T cell help is required for the generation of a CTL response. However, primary CD8$^+$ T cell responses to *L. monocytogenes* (Sun and Bevan, 2003) and lymphocytic

Figure 11. Cytolytic activity is mediated by perforin but not by Fas

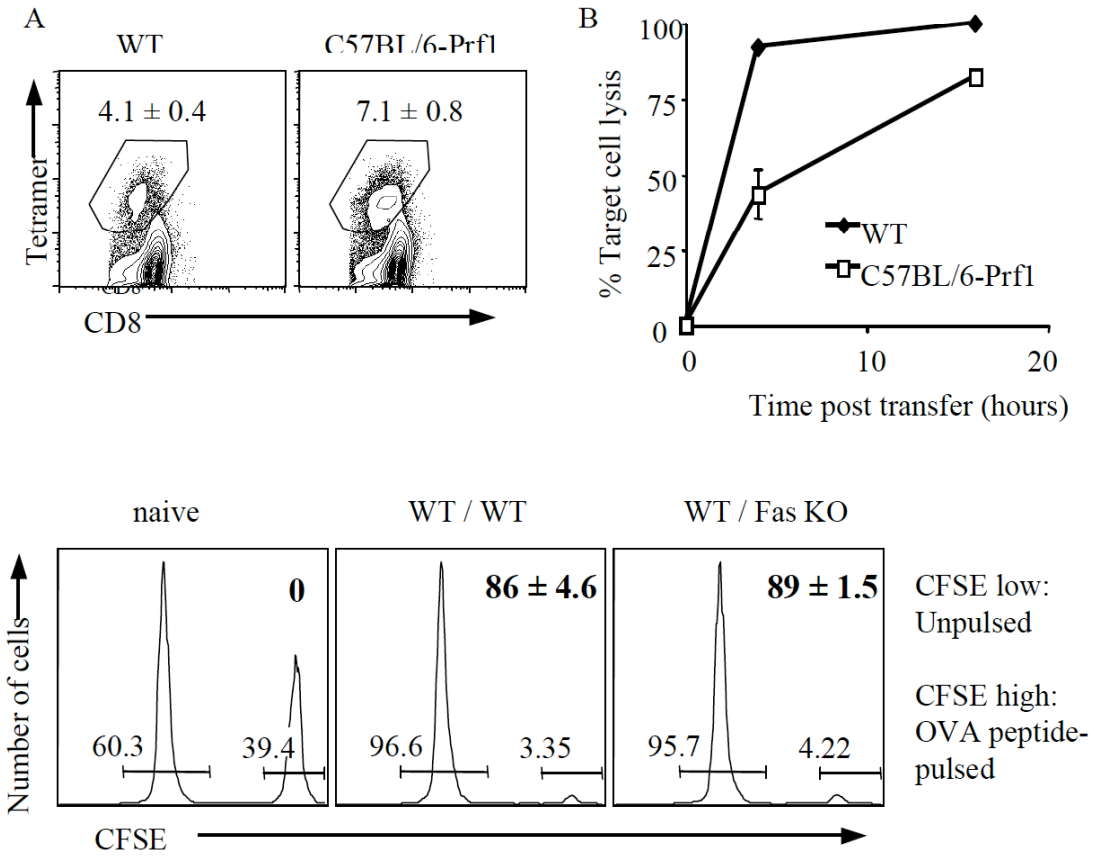


Figure 11. Perforin-deficient mice have defective cytolytic activity as determined by an *in vivo* CTL assay. (A) Frequency of tetramer$^+$ cells (out of CD3$^+$CD8$^+$population). Data are average ± standard deviation for 3 mice per group. (B) Perforin-deficient mice have decreased cytolytic activity. (C) Cytolytic activity in naïve (left panel) or immunized (center and right panels) mice that received WT (center) or Fas KO (right) OVA peptide-pulsed cells. Numbers in top right represent average ± standard deviation of cytolysis for WT or Fas KO targets (n = 3 mice per group). Perforin-KO or Fas KO experiments were each repeated twice with similar results.

Adapted from KA Jordan et al, Infection and Immunity 2009.

Figure 12. CD4$^+$ T cell help is required for optimal expansion of tetramer$^+$ CD8$^+$ T cells in response to CPS-OVA immunization

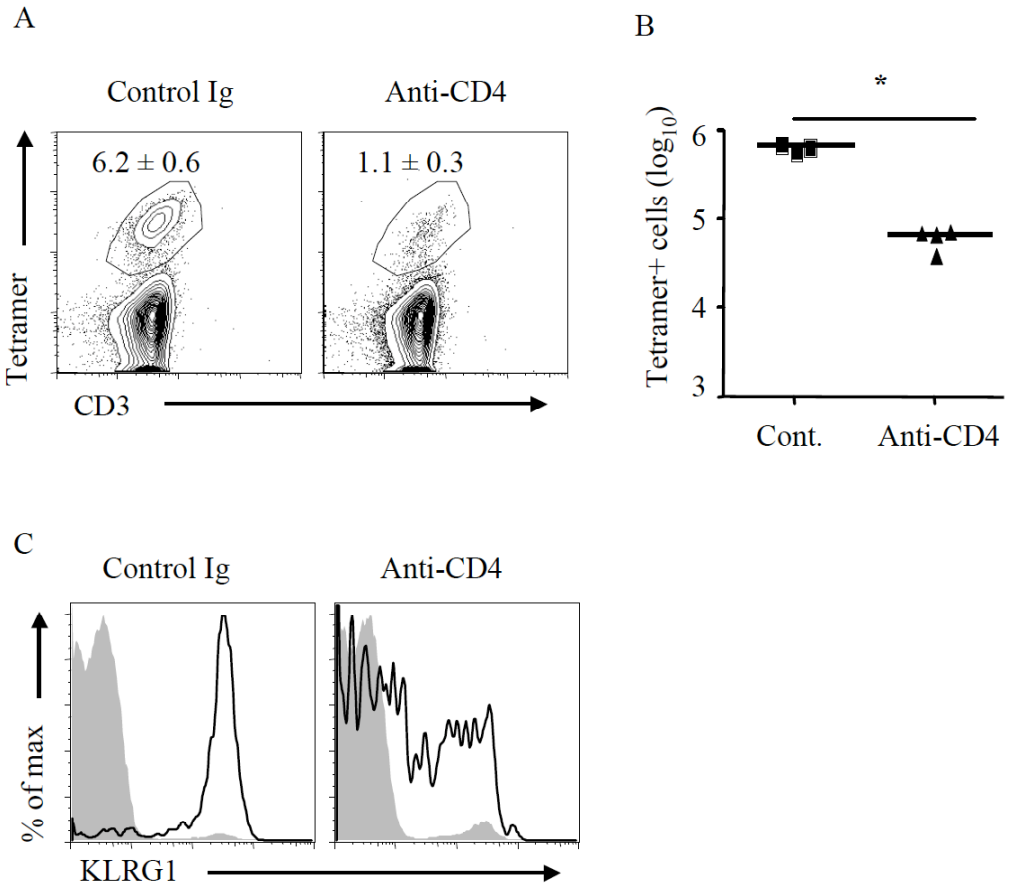


Figure 12. CD4$^+$ T cells provide help for CD8$^+$ T cells in response to CPS-OVA immunization. Mice were depleted of CD4$^+$ T cells 1 week prior to immunization with CPS-OVA and results were analyzed 8 days later. (A) Frequency of tetramer$^+$ cells is reduced (average of 4 mice from one representative experiment ± standard deviation). (B) Number of tetramer$^+$ per spleen. (C) Expression of KLRG1 is reduced on tetramer$^+$ cells generated in the absence of CD4$^+$ help (tetramer- cells gray histogram; tetramer$^+$ cells open histogram). CD4$^+$ T cell depletion was verified by staining for CD4 when mice were analyzed; depletion was consistently > 95%.

Adapted from KA Jordan et al, Infection and Immunity 2009.

choriomeningitis virus (LCMV) (Shedlock and Shen, 2003) are $CD4^+$ T cell-independent. Following infection with a replicating strain of *T. gondii*, $CD4^+$ T cell help is not required for the generation of CD8 effector responses (Lutjen et al., 2006). To address whether $CD4^+$ T cell help was required in the absence of parasite replication, mice were depleted of $CD4^+$ T cells one week prior to immunization with two doses of 0.5 mg anti-CD4 antibody. One week following immunization, CD4-depleted mice demonstrated a decreased frequency as well as total number of OVA-specific $CD8^+$ T cells (Figure 12A-B). However, the OVA-specific T cells generated in the absence of $CD4^+$ T cell help were still capable of making IFN-γ in response to OVA protein stimulation (2.07 ± 0.8 vs. 1.15 ± 0.36 ng/ml by ELISA, no significant difference). Expression of KLRG1 was decreased on antigen-specific $CD8^+$ T cells following CD4 depletion (Figure 12C; 76.3 ± 3.2% vs. 21.77 ± 4% for CD4-depleted mice, p=0.0004). Additionally, $CD8^+$ effector T cells generated without $CD4^+$ T cell help were capable of killing peptide-pulsed target cells as demonstrated by an *in vivo* CTL assay, though the rates of cytolysis were decreased (data not shown). This decreased level of killing is most likely related to the lower frequency of antigen-specific CTL at this time, and as shown in Figure 10D, there is a positive correlation between frequency of $tetramer^+$ cells and levels of cytolysis.

3.6 Discussion

The studies presented here build on the original report using the cps-II parasites and establish that this replication-deficient strain induces a protective response that is dependent on $CD4^+$ and $CD8^+$ T cells. We took advantage of a parasite expressing OVA, which allowed us to carefully and precisely characterize the numbers and frequencies of

these antigen-specific $CD8^+$ T cells, and provided new information about phenotype and cytolytic function of these cells. While these studies were in progress, the Yap laboratory proposed that antigen-specific T cells induced by cps-II immunization are heterogeneous and can be subdivided into four sub-populations based on their expression of KLRG1 and CD62L (Wilson et al., 2008). FI is $CD62L^{high}KLRG1^-$, FII is $CD62L^{low}KLRG1^-$, FIII is $CD62L^{low}KLRG1^+$, and FIV is $CD62L^{high}KLRG1^+$, with the FIII population being most enriched for granzyme B^+ IFN-γ-producing effector cells. Our results using a model antigen, where *T. gondii*-specific cells can be precisely identified using a tetramer, suggest that $tetramer^+$ cells are almost entirely $CD62L^{lo}$ and $KLRG1^+$, or falling entirely into Fraction III as defined by the Yap group. Interestingly, the kinetics of Fraction III cells most closely resembled the expansion of the $tetramer^+$ $CD8^+$ T cell population we observed using the CPS-OVA immunization model, and further work will be needed to examine whether these sub-populations are truly parasite-specific, and if so, what factors influence their development.

One concern with these studies was that the OVA-specific response might not represent the T cell response to bona fide *T. gondii* antigens. However, recent work from two laboratories has identified endogenous epitopes of *T. gondii* in B10.D2 and BALB/c mice (Blanchard et al., 2008; Frickel et al., 2008). In both of those studies, these immundominant antigens were part of secreted molecules and so are likely to be presented in a similar fashion to the model antigen used here and by others (Dzierszinski et al., 2007; Pepper et al., 2004), suggesting that the findings presented in this work will be representative of the polyclonal response *to T. gondii*. The identification of

endogenous $CD8^+$ T cell epitopes in mice on the more commonly studied C57BL/6 strain will be fortuitous and will aid further study into this area.

One of the strengths of this experimental system is the ability to identify long-lived populations of parasite-specific cells. The pattern of expression of some activation and memory markers differed from what has been seen in other models where the duration of antigen exposure is limited, such as in a model of acute LCMV infection (Kaech et al., 2003). In that system, for example, antigen-specific cells downregulate expression of the IL-7Rα quickly following activation, and one week after infection nearly all antigen-specific cells are negative for IL-7Rα. The functional consequences of dynamic IL-7Rα are relevant because studies in other systems have correlated the expression of this marker with the capacity to form $CD8^+$ T cell memory, and in LCMV infection nearly 100% of cells regain expression of that marker by day 40 (Kaech et al., 2003). This is in contrast with what was seen after CPS-OVA immunization, where only approximately two-thirds (67.7 ± 2.9%) of OVA-specific splenocytes express high levels of IL-7Rα 75-90 days after immunization. However, recent reports on antigen-specific $CD8^+$ T cells in *Trypanosoma cruzi*-infected, drug-cured mice suggest that the conversion of $CD8^+$ T cells to a memory phenotype occurs very slowly (Bixby and Tarleton, 2008; Bustamante et al., 2008). One implication of this analysis is that the combination of markers commonly used to track memory populations may have to be tailored to individual pathogens and may not be applicable to every infection.

The initial expectation was that a replicating form of *T. gondii,* that induces tissue damage and produces increasing amounts of antigen, would promote a more rapid and

quantitatively greater $CD8^+$ T cell response than that generated by the CPS parasites. Unexpectedly, these studies revealed that CPS-OVA induced an adaptive $CD8^+$ T cell response with faster kinetics than reported with replicating parasites (Kwok et al., 2003; Lutjen et al., 2006). One potential explanation for this difference is that replicating parasites produce factors that limit host cell proliferation and cytokine responses (Candolfi et al., 1994; Khan et al., 1995). These effects would presumably be muted if the parasite cannot replicate. Further studies will be needed to characterize early immune responses to the non-replicating parasite, including how the innate and inflammatory response contributes to the robust generation of the $CD8^+$ T cell response following CPS-OVA infection. While we did not directly measure IL-12 levels, there has been one report that CPS parasites can induce high levels of IL-12p70 at the site of infection as well as the spleen (Gigley et al., 2009), consistent with the idea that this cytokine is a major determinant promoting $CD8^+$ T cell responses during CPS-OVA infection. This idea will be further addressed in Chapter 4.

Previous analysis of cytotoxic responses induced by *T. gondii* required that splenocytes from infected mice or those immunized with STAg undergo expansion in the presence of exogenous cytokines for 6-7 days, which may have influenced their effector function (Hakim et al., 1991). In contrast, direct *ex vivo* staining 7 days following CPS-OVA immunization showed that nearly 100% of OVA-specific cells expressed the cytolytic effector molecule granzyme B, and an *in vivo* CTL assay confirmed that these cells were cytolytic. While cytolytic activity was decreased in perforin-deficient mice, these mice were still able to generate a robust antigen-specific $CD8^+$ T cell response to CPS-OVA and could mediate a degree perforin-independent cytolysis.

As discussed earlier, the requirement for $CD4^+$ T cell help in the generation of $CD8^+$ effector T cell responses can differ depending on the type of infection. In studies with *T. gondii*, it was previously demonstrated that CD4 KO mice had decreased numbers of antigen-specific $CD8^+$ T cells as demonstrated by a precursor cytotoxic T lymphocyte assay (Casciotti et al., 2002). This defect was only seen at late time points following *T. gondii* infection, however it is possible that defects were also present earlier during infection but were not detectable by the methods available at that time. In another model of *T. gondii* infection, $CD4^+$ T cell help was required to maintain antigen-specific $CD8^+$ T cells in the brain during chronic infection (Lutjen et al., 2006), but did not affect generation of acute CTL responses. Studies with another type of protozoal infection, *Trypansoma cruzi*, demonstrated that $CD4^+$ T cell help was required during the primary response to immundominant epitopes (Padilla et al., 2007). The findings presented here might thus be influenced by the fact that the H-$2K^b$-restricted SIINFEKL epitope is known to be immunodominant. While $CD4^+$ T cell production of IL-2 has been regarded as the most likely mechanism that contributes to the development of $CD8^+$ T cell responses, there may be an alternative explanation. One possibility is that $CD4^+$ T cell help is required to license dendritic cells to promote optimal $CD8^+$ T cell effector and memory responses, something that has been shown with HSV-1 infection (Smith et al., 2004). Regardless, these results suggest that in a vaccine setting, both $CD4^+$ and $CD8^+$ T cell epitopes should be targeted in order to drive a protective response.

Chapter 4: c-Rel in the Generation of $CD8^+$ T cell Responses

4.1 Introduction

The NF-κB transcription factor c-Rel is exclusively expressed in immune cells and plays a role in numerous cellular functions including proliferation, survival and production of chemokines and cytokines. c-Rel has also been implicated in the regulation of multiple genes that are involved in innate and adaptive immune responses to the intracellular protozoan parasite *T. gondii*, in particular IL-12. Previous studies from this laboratory have defined a critical role for c-Rel in resistance to *T. gondii*, though this earlier work did not clarify whether $CD8^+$ T cells had an intrinsic requirement for c-Rel, or if this transcription factor was required for maintenance of $CD8^+$ memory T cell populations. To better understand how this transcription factor controls the $CD8^+$ T cell response to this parasite, WT and c-$Rel^{-/-}$ mice were challenged with a replication-deficient strain of *T. gondii* that expresses the model antigen ovalbumin. This thesis chapter will discuss my work looking at the primary $CD8^+$ T cell responses as well as memory responses, and the T cell-intrinsic versus -extrinsic responses for the transcription factor c-Rel. These results indicate that in this infection model, the major influence of c-Rel in generation of $CD8^+$ T cell responses is through its regulation of the inflammatory environment, rather than playing a substantial T cell-intrinsic role.

4.2 c-Rel is required for optimal antigen-specific $CD8^+$ T cell expansion and resistance to secondary challenge

Because c-$Rel^{-/-}$ mice are susceptible to infection with replicating strains of *T. gondii* (Mason et al., 2004a), a model was used in which a replication-deficient strain of *T. gondii* induces protective immunity in WT mice, and this depends on $CD8^+$ T cells at the time of secondary challenge (Fox and Bzik, 2002; Jordan et al., 2009). Moreover, as introduced in the previous chapter, since this vaccine strain expresses the model antigen ovalbumin, the endogenous $CD8^+$ T cell response to OVA can also be tracked using MHC class I tetramers. To first assess whether c-Rel was required for the ability of CPS-OVA to induce protective immunity, groups of WT and c-$Rel^{-/-}$ mice were immunized with 10^5 CPS-OVA parasites and challenged with virulent RH-OVA 30 days later. Naive WT and c-$Rel^{-/-}$ mice succumbed to infection between 8 and 9 days following infection (Figure 13). WT mice that had been immunized were able to control this challenge. In contrast, immunized c-$Rel^{-/-}$ mice did not survive, though there was a significant and reproducible delay in time to death in comparison to challenged naïve c-$Rel^{-/-}$ mice (Figure 13, $p = 0.0014$ by one-way ANOVA). At the time of death large numbers of free parasites could be found in the peritoneal cavities of immunized and challenged c-$Rel^{-/-}$ but not WT mice, and all naïve challenged mice regardless of genotype (data not shown).

In this model, protective immunity is primarily dependent on $CD8^+$ T cells, thus studies were performed to assess if the absence of c-Rel affected the generation of an antigen-specific $CD8^+$ T cell population. WT and c-$Rel^{-/-}$ mice were immunized with CPS-OVA and the antigen-specific response was tracked using a tetramer reagent that

Figure 13. Immunization does not provide c-Rel$^{-/-}$ mice substantial protection from rechallenge

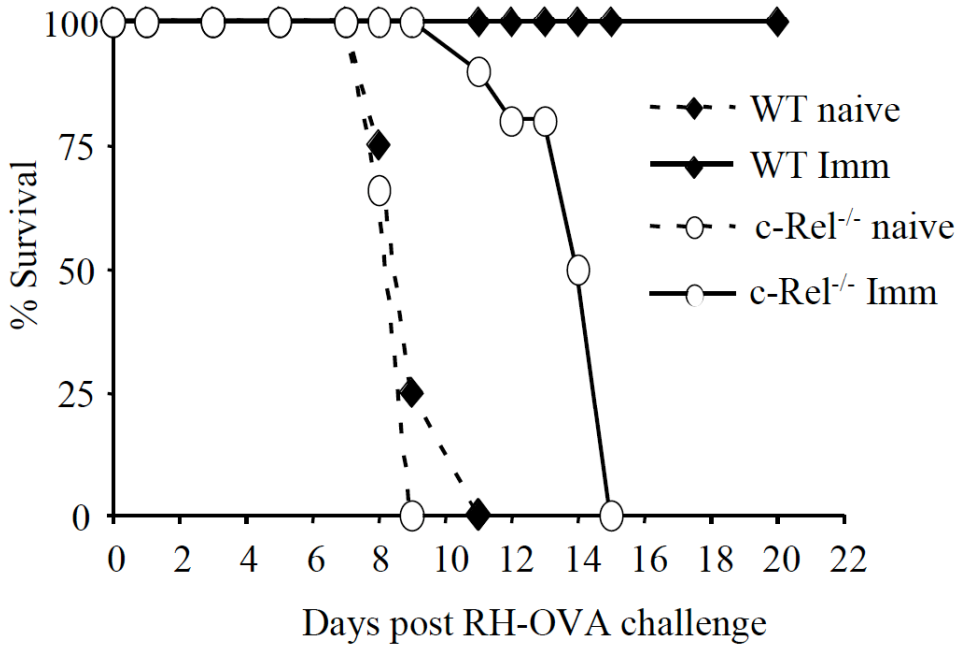


Figure 13. WT and c-Rel$^{-/-}$ mice were immunized with 10^5 CPS-OVA parasites. 30 days later, naïve or immunized mice were challenged with 10^3 RH-OVA parasites and survival was monitored. Data represent combined results from 2 similar experiments. WT vs. c-Rel$^{-/-}$ immunized mice, $p = 0.0014$ by one-way ANOVA.

Adapted from KA Jordan et al., International Immunology (under revision).

Figure 14. c-Rel$^{-/-}$ mice have a reduced expansion of antigen-specific CD8^{+} T cells in response to CPS-OVA

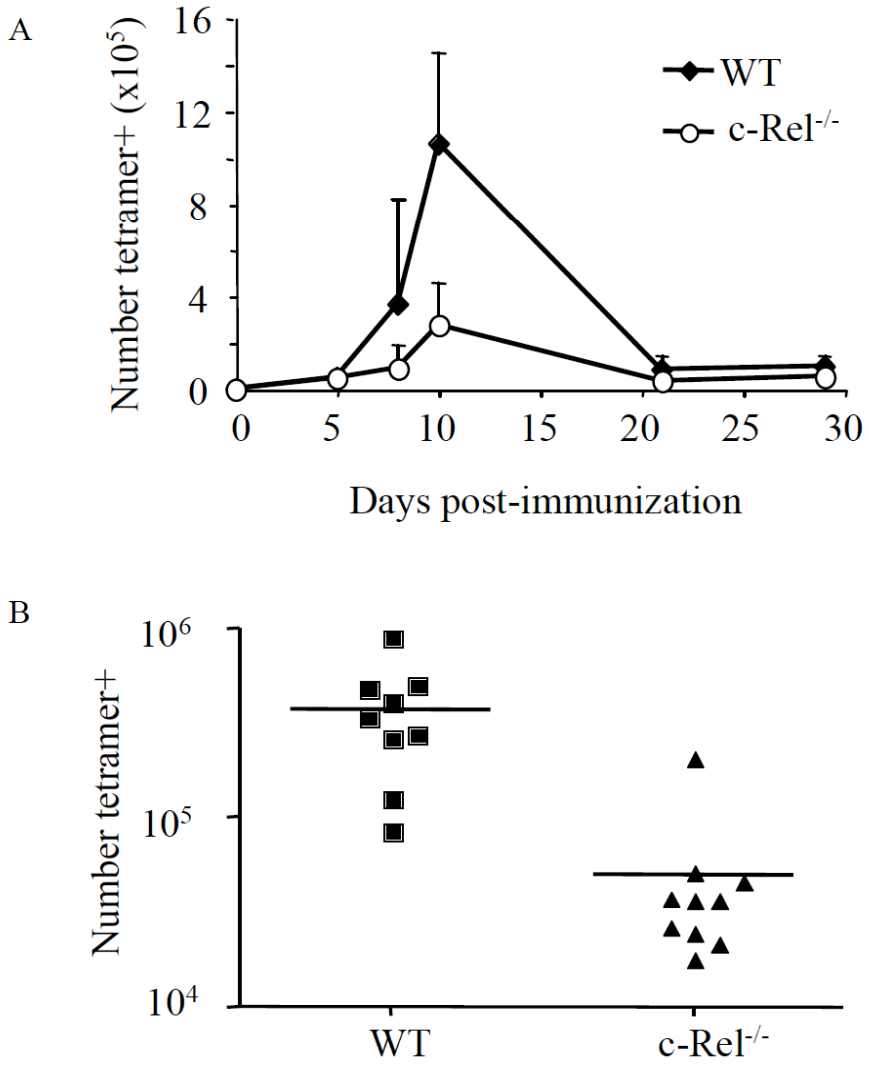


Figure 14. Antigen-specific responses of WT and c-Rel$^{-/-}$ mice were tracked using tetramer staining. (A) Number of tetramer-specific cells in the spleen. Data represent mean ± standard deviation for at least 3 mice per group from 1 of 2 representative experiments. (B) Number of tetramer^{+} cells per spleen in WT and c-Rel$^{-/-}$ mice at day 8. Each symbol represents one individual mouse ($p = 0.0006$).

Adapted from KA Jordan et al., International Immunology (under revision).

recognizes T cell receptors specific for the immunodominant SIINFEKL epitope derived from ovalbumin, in the context of MHC I H-2K^b. Consistent with the data in Chapter 3, analysis of the primary response revealed that WT mice displayed a robust primary expansion (Figure 14A). Also consistent with their increased susceptibility to rechallenge, c-Rel$^{-/-}$ mice exhibited a decrease in frequency as well as number of OVA-specific CD8$^+$ T cells at all time-points examined in the first 30 days of infection (Figure 14 and data not shown). The combined results from three experiments analyzed 8 days following immunization demonstrated that both the frequencies (7.175% ± 0.76 vs 1.8% ± 0.35, $p < 0.0001$) as well as absolute numbers ($3.76 \times 10^5 \pm 8.0 \times 10^4$ vs. $5.04 \times 10^4 \pm 1.76 \times 10^4$; $p = 0.0006$) of antigen-specific splenocytes were significantly reduced in c-Rel$^{-/-}$ mice compared to WT control mice (numbers shown in Figure 14B). Similar results were seen at this time-point in the PECs as well as the blood (data not shown). Thus, during the primary response to CPS-OVA immunization, c-Rel is required for the optimal development of antigen-specific CD8$^+$ T cells, and this correlates with insufficient protection during secondary challenge.

4.3 c-Rel is not required for effector function but is required to upregulate the activation marker KLRG1

Although the data shown in Figure 14 indicate that c-Rel influences the magnitude of the CD8$^+$ T cell response, studies were performed to determine if the antigen-specific cells generated in the c-Rel$^{-/-}$ mice were functionally competent. Peritoneal cells from immunized WT and c-Rel$^{-/-}$ mice taken 8 days post-immunization were stimulated *ex vivo* with OVA peptide. As TCR stimulation leads to down-regulation

of tetramer binding (Drake et al., 2005; Kao et al., 2005), dual staining for cytokines and OVA tetramer was not successful. However, in response to anti-CD3 (not shown) or OVA peptide stimulation, both WT and c-Rel$^{-/-}$ cells were capable of making IFN-γ and TNF-α. This conclusion was drawn by comparing the frequency of cells that produced IFN-γ in response to OVA peptide stimulation *ex vivo* (Figure 15B) to the frequency of tetramer^{+} cells (Figure 15A) in the tissues of individual mice (summarized in Figure 15C). Of the cells that were making IFN-γ in response to OVA peptide, there was no difference in the MFI (535 ± 21 vs. 491.7 ± 23, p = 0.3). Similar frequencies of cells that made IFN-γ could also produce TNF-α *ex vivo* (32.7 ± 5.4 vs. 32.6 ± 7.7; n = 3/ group from one representative experiment, no significant difference), and the MFI of TNF-α from double-producers did not differ between WT and c-Rel$^{-/-}$ mice (468.7 ± 2.9 vs. 472 ± 4.2; n = 3/ group from one representative experiment, no significant difference). Splenocytes and PECs were also stimulated with soluble *T. gondii* antigens (STAg), and IFN-γ production was measured by ELISA. Similarly, less IFN-γ was found in supernatants from c-Rel$^{-/-}$ mice as compared to WT mice (data not shown); however, this is consistent with the reduced numbers of antigen-specific CD8^{+} T cells generated following immunization. Moreover when an *in vivo* cytolysis assay using transferred OVA peptide-pulsed target cells was used to determine if c-Rel$^{-/-}$ antigen-specific CD8^{+} T cells could mediate cytotoxicity, no defect was observed in the ability of c-Rel$^{-/-}$ mice to kill the transferred target cells (Figure 15D). However, as shown in Figure 9C (Chapter 3), maximum cytolytic activity is reached at 16 hours, when this assay was analyzed. If these mice had been examined earlier it is likely the levels of cytolysis would have been

lower, given the reduced frequency of tetramer^{+} cells in c-Rel$^{-/-}$ mice (7.6-fold for the data shown in Figure 15D).

WT and c-Rel$^{-/-}$ splenocytes were next subjected to tetramer staining and compared to CD8^{+} T cells from naïve WT mice (Figure 16A). Phenotypic characterization of antigen-specific splenocytes revealed no observable differences between tetramer populations of WT and c-Rel$^{-/-}$ mice in their expression of CD62L, CD122 or Granzyme B. The only significant phenotypic difference at the day 8 time point was the expression of KLRG1; though originally described as an NK cell activation marker, it has since been shown that KLRG1 is upregulated on CD4^{+} and CD8^{+} T cells following infection (Beyersdorf et al., 2001; Robbins et al., 2003). In various models of infection and inflammation its expression has been linked directly to the ability of CD8^{+} T cells to respond to IL-12 (Joshi et al., 2007; Kaech et al., 2003; Wilson et al., 2008). Significantly fewer tetramer^{+} cells from the spleen (86.6 ± 2.7 vs. 36.6 ± 5.6; $p < 0.0001$) or PECs (Figure 16B, 75.9± 2.8 vs. 35.5 ± 5.7; $p < 0.0001$) of c-Rel$^{-/-}$ mice expressed KLRG1, suggesting that the CD8^{+} T cells induced by CPS-OVA in c-Rel$^{-/-}$ mice are either not able to respond to IL-12, or that they are not receiving enough of this cytokine. Preliminary analysis of T-bet, a transcription factor linked to IL-12 production, did not indicate any defect in c-Rel$^{-/-}$ tetramer^{+} cells (MFI of 399 versus 418 in one experiment), though this should be analyzed more thoroughly in future experiments.

Figure 15. OVA-specific CD8$^+$ T cells from c-Rel$^{-/-}$ mice are not defective in cytokine production or cytolysis

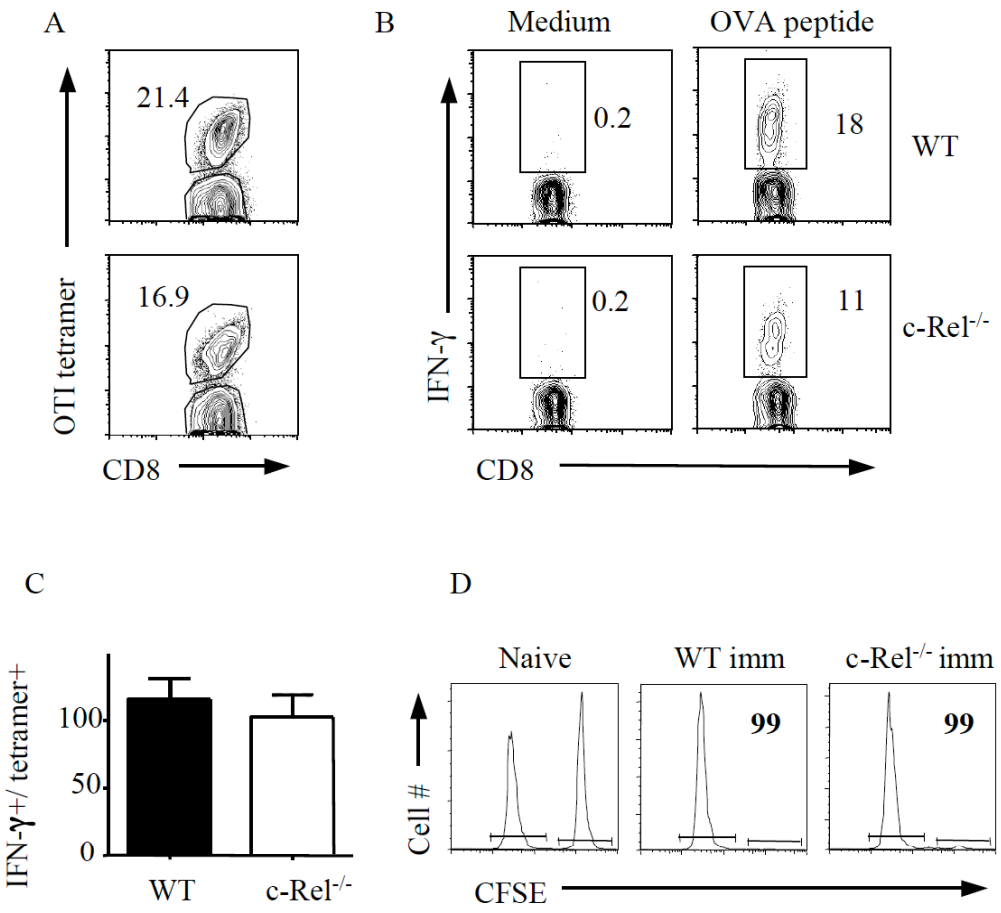


Figure 15. WT and c-Rel$^{-/-}$ mice were analyzed 8 days after immunization with 10^5 CPS-OVA. (A) Frequency of tetramer$^+$ cells in the PECs, out of CD3$^+$8$^+$ cells. (B) Frequency of IFN-γ$^+$ cells out of CD8$^+$ cells from the same mice as in part A, stimulated for 5 hours with OVA peptide in the presence of BFA. (C) Values were calculated by dividing the frequency of (B) over (A). (D) Mice were assessed for cytolytic activity by an *in vivo* CTL assay. Plots are gated on CD45.1 donor cells and cytolysis (loss of CFSE high cells) was calculated as indicated in Figure 5, and is indicated in. the upper right corner of each plot.

Adapted from KA Jordan et al., International Immunology (under revision).

Figure 16. Phenotypic characterization of tetramer$^+$ splenocytes from WT and c-Rel$^{-/-}$ mice

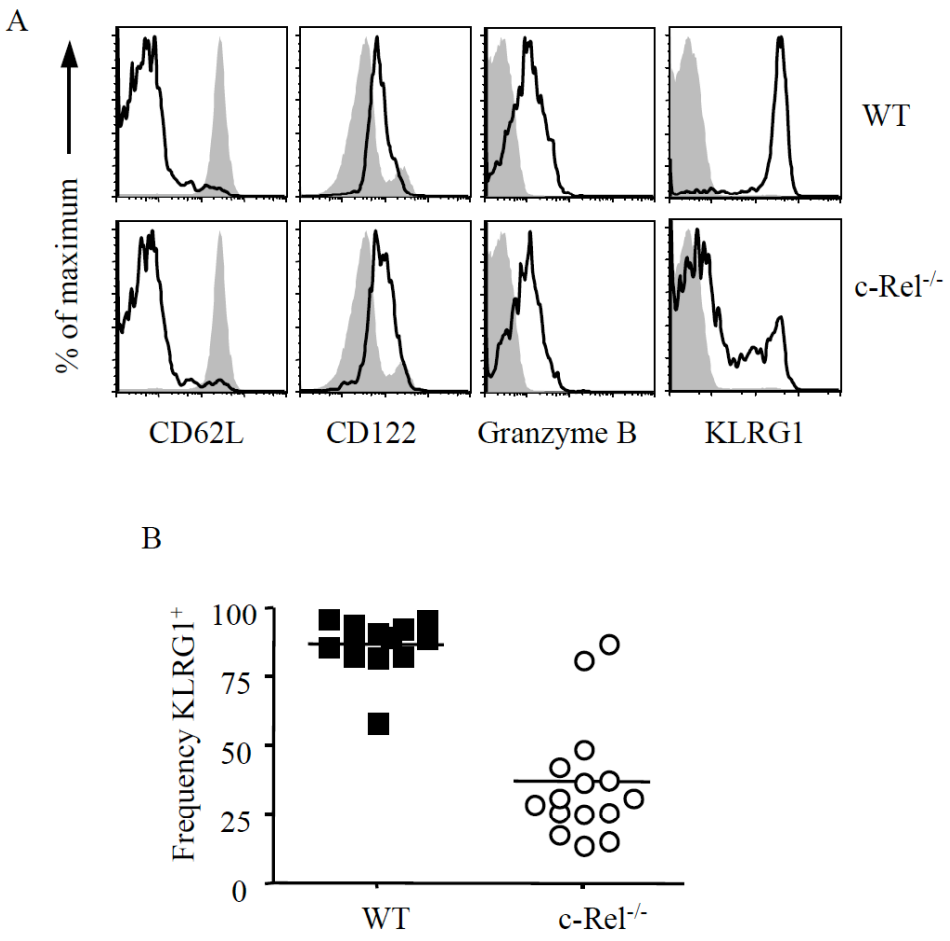


Figure 16. WT and c-Rel$^{-/-}$ splenocytes were analyzed 8 days after immunization with 10^5 CPS-OVA. (A) Histograms represent tetramer$^+$ cells (open) compared to naïve (filled) CD3$^+$CD8$^+$ cells for the markers noted on the bottom. (B) Frequency of tetramer$^+$ cells that are KLRG1$^+$ is significantly reduced in c-Rel$^{-/-}$ mice (each symbol represents one mouse; data are combined from 3 similar experiments).

Adapted from KA Jordan et al., International Immunology (under revision).

4.4 A T-cell extrinsic function for c-Rel in the regulation of $CD8^+$ T cell responses

In parallel, studies using a replication competent strain of *T. gondii* expressing OVA (Pru-OVA) were used to distinguish T cell-intrinsic versus T cell-extrinsic requirements for c-Rel. c-$Rel^{-/-}$ mice were crossed with OTI transgenic mice to generate TCR transgenic $CD8^+$ T cells lacking c-Rel. WT and c-$Rel^{-/-}$ OTI cells were transferred into Thy-disparate recipient mice that were infected one day later with Pru-OVA. When T cell responses were analyzed between 7-9 days following infection, WT and c-$Rel^{-/-}$ OTI cells expanded similarly in terms of frequency as well as number (Figure 17A-B). This finding indicates that, in this system of infection-induced proliferation, $CD8^+$ T cells themselves do not intrinsically require c-Rel. It further suggests that other factors in the c-$Rel^{-/-}$ environment, such as pro-inflammatory cytokines or $CD4^+$ T cell help, might contribute to the decreased generation of tetramer cells in c-$Rel^{-/-}$mice. Another potential explanation for the defective $CD8^+$ T cell responses was a lack of $CD4^+$ T cell help. While adoptive transfer studies to see if $CD4^+$ T cell help from WT mice could restore $CD8^+$ T cell effector responses in c-$Rel^{-/-}$ mice were not performed, it is likely that defects in c-$Rel^{-/-}$ $CD4^+$ T cells contributed to the poor $CD8^+$ T cell responses, given what is known about their reduced capacity to proliferate and to produce IL-2. The $CD4^+$ T cell depletion studies described in Chapter 3 did define a requirement for $CD4^+$ T cell help during the effector response to immunization, suggesting that $CD4^+$ T cell help could be one contributing factor in the sub-optimal $CD8^+$ T cell expansion of c-$Rel^{-/-}$ mice.

Figure 17. A T cell-extrinsic function for c-Rel during the immune response to *T. gondii*

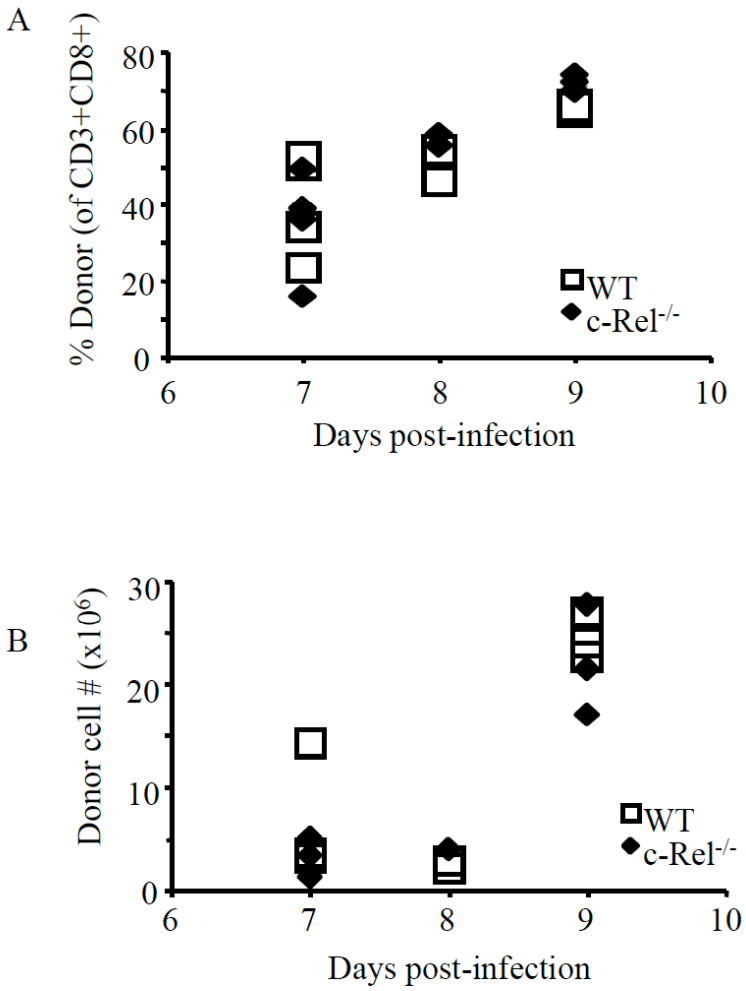


Figure 17. WT and c-Rel$^{-/-}$ OTI cells were adoptively transferred into Thy-disparate mice. One day later, mice were infected with Pru-OVA. Kinetics of the antigen-specific donor cell response was tracked over time in the spleen. Similar expansion of WT and c-Rel$^{-/-}$ OTI cells was noted in terms of frequency (A) as well as number (B).

Adapted from KA Jordan et al., International Immunology (under revision).

Figure 18. Inflammatory environment regulates CD8$^+$ T cell expression of KLRG1

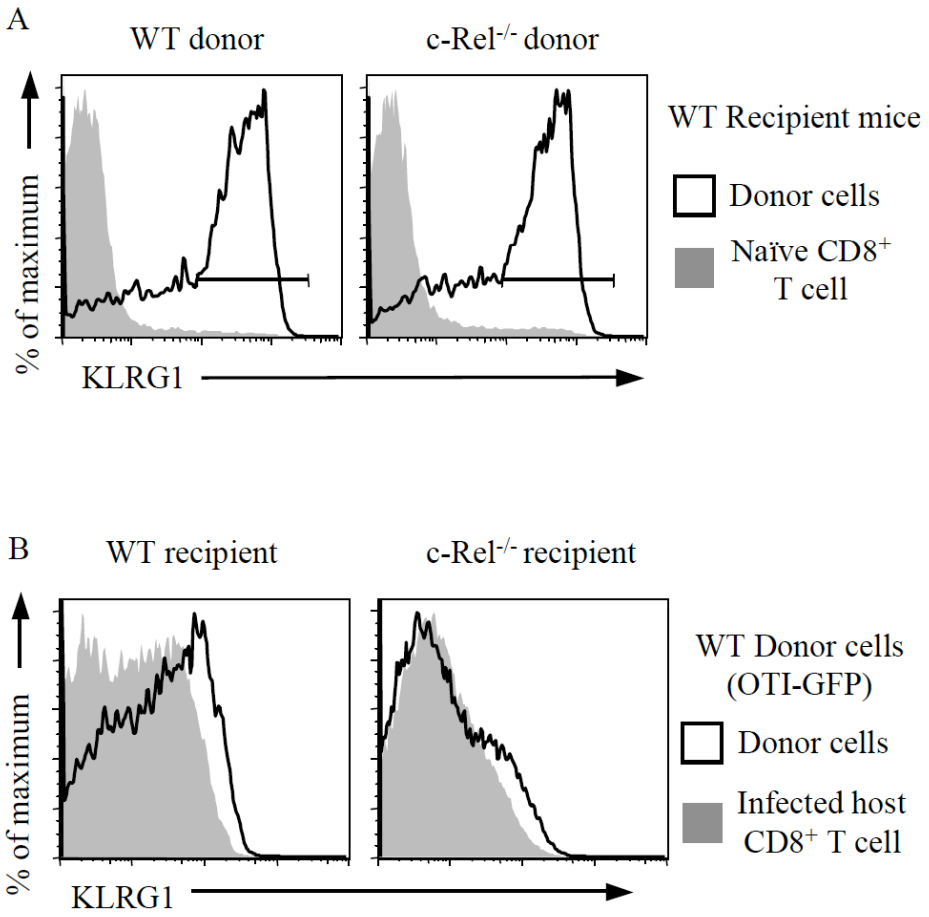


Figure 18. (A) WT and c-Rel$^{-/-}$ OTI cells were adoptively transferred into Thy-disparate mice. One day later, mice were infected with Pru-OVA and responses of the antigen-specific donor cells were tracked. Histograms represent donor (open histogram, CD8$^+$Thy1.2$^+$) or naive CD8$^+$ T cells (filled histogram, CD8$^+$Thy1.1$^+$). (B) WT OTI-GFP cells (open black histogram) were adoptively transferred into WT or c-Rel$^{-/-}$ recipients and analyzed for KLRG1 expression compared to host CD8$^+$ T cells (shaded gray histogram). A and B were analyzed at day 7 post-infection with Pru-OVA.

Adapted in part from KA Jordan et al., International Immunology (under revision).

The WT host environment also impacted the phenotype of the transferred antigen-specific OTI cells, because the c-Rel$^{-/-}$ OTI cells were able to express similar levels of KLRG1 compared to WT cells (Figure 18A). This suggests that the inability of cells to express this marker in c-Rel$^{-/-}$ mice is not intrinsic to the T cell but is dependent on accessory cells. This was further supported by reciprocal adoptive transfer experiments where WT OTI cells were transferred into WT and c-Rel$^{-/-}$ recipients, and infected with Pru-OVA. Donor cells were analyzed at day 8 and in this system WT OTI-GFP cells demonstrated decreased expression of KLRG1 following adoptive transfer into a c-Rel$^{-/-}$ recipient (Figure 18B). To further explore the defective environment, the ability to produce cytokines *ex vivo* in response to OVA protein and OVA peptide was analyzed. Figure 19 demonstrates that WT recipient APC (top) could support production of IFN-γ by these WT OTI-GFP T cells, while c-Rel$^{-/-}$ recipient APC (bottom) were defective when T cells were stimulated with OVA protein ($p = 0.02$) even when IL-2 was added ($p= 0.02$). The c-Rel-/- APC could only induce potent IFN-γ production when the cells were stimulated with OVA peptide (p = .n.s. for OVA peptide, even with IL-2). Together, these studies clearly indicate that the c-Rel$^{-/-}$ environment does not provide an optimal environment for the activation of WT CD8^{+} T cells.

4.5 IL-12 and regulation of CD8^{+} T cell responses

One possible candidate that may explain the defective CD8^{+} T cell responses in c-Rel$^{-/-}$ mice is IL-12. This cytokine is known to contribute to the primary expansion of CD8^{+} T cells in response to infection with a number of organisms, serving as “signal 3”

Figure 19: WT CD8^{+} T cells are defective in cytokine production in a c-Rel$^{-/-}$ host

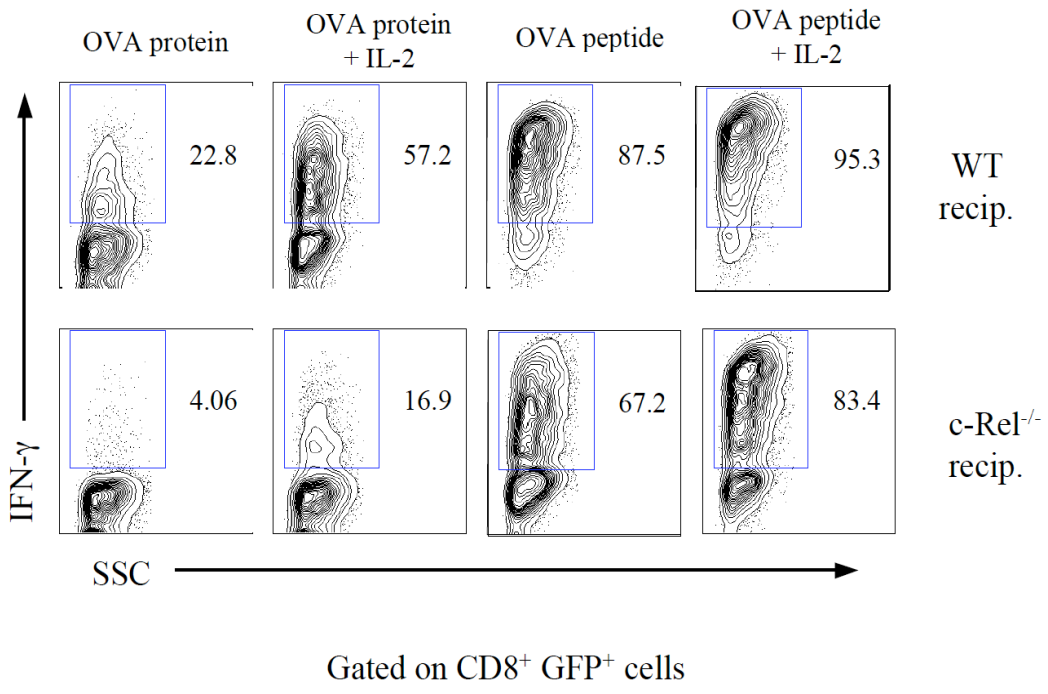


Figure 19. WT cells can't respond to OVA protein stimulation with c-Rel$^{-/-}$ APCs. WT and c-Rel$^{-/-}$ mice received WT OTI-GFP cells and were subsequently infected with Pru-OVA. 7 days post infection splenocytes were isolated and restimulated *ex vivo* with OVA protein and OVA peptide, in the presence or absence of exogenous IL-2. Cells were then stained for GFP and the presence of intracellular IFN-γ. Plots show WT recipients (top row) and c-Rel$^{-/-}$ recipients (bottom row) and are gated on CD8^{+} donor^{+} cells. Numbers represent average frequency of IFN-γ-producing cells (n = 2).

Figure 20. Depletion of IL-12 decreases expansion of tetramer$^+$ population and decreases expression of KLRG1

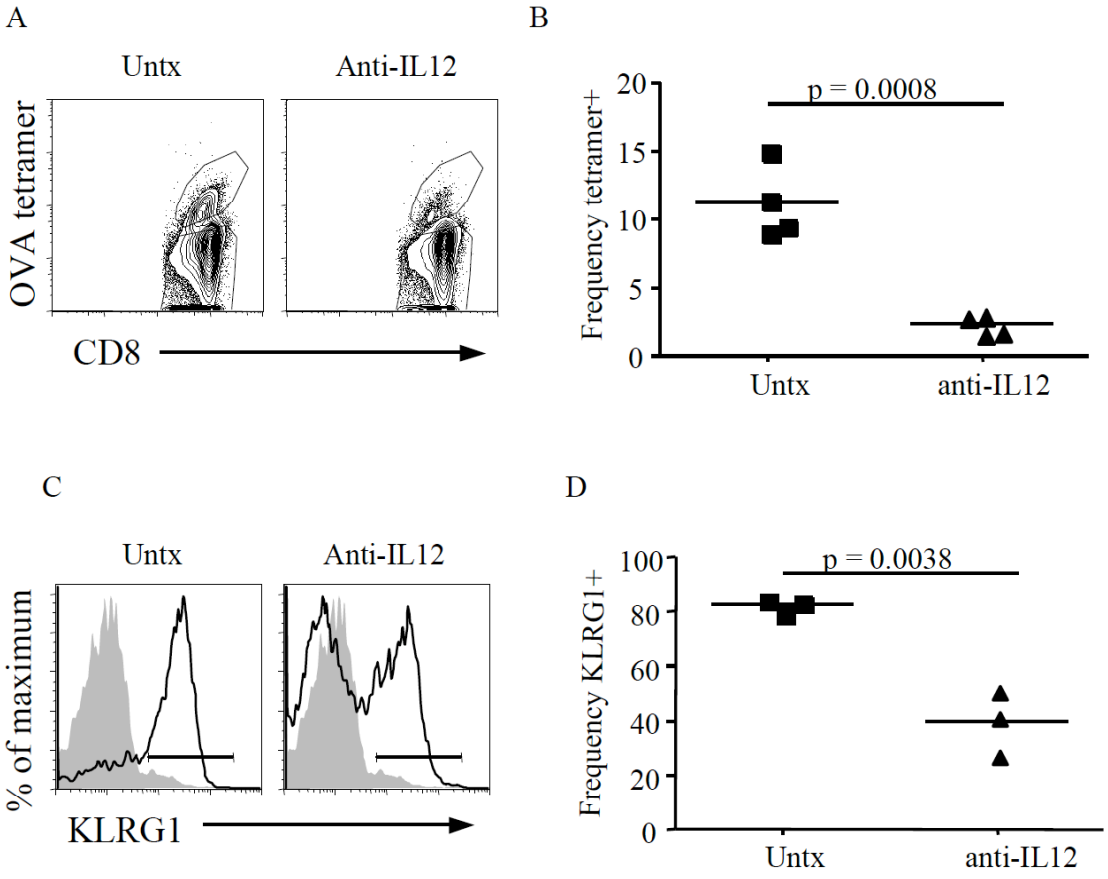


Figure 20. WT mice were treated with PBS (untx) or with 2 doses of anti-IL12, and immunized. Spleens were analyzed 8 days following CPS-OVA immunization. (A) Representative tetramer staining. (B) Significantly fewer tetramer$^+$ cells were generated in the spleen of mice treated with anti-IL12. (C) Representative KLRG1 staining, gated on naïve CD8$^+$ T cells (shaded gray) or tetramer$^+$ cells (black line). (D) Significantly lower expression of KLRG1 was found on tetramer$^+$ cells from anti-IL12 treated mice.

Adapted in part from KA Jordan et al., International Immunology (under revision).

in addition to the obligatory TCR/MHC plus co-stimulation required to generate an antigen-specific response (Pearce and Shen, 2007). Moreover, c-Rel$^{-/-}$ mice have been reported to have defects in the acute production of IL-12 at the site of infection (Mason et al., 2004a). Therefore, studies were performed to assess the role of IL-12 in the development of antigen-specific CD8^{+} T cells in response to CPS-OVA. WT mice were treated with 2 doses of anti-IL-12p40 antibody on day -1 and day 2 of infection with CPS-OVA and this resulted in decreased generation of tetramer^{+} cells in the spleen (11.3 ± 1.4% vs. 2.4 ± 0.3% ($p = 0.0008$); Figure 20A-B). IL-12 depletion also caused a significant decrease in the expression of KLRG1 on tetramer^{+} cells in the spleen (82.5 ± 1.6% vs. 39.8 ± 6.9% ($p = 0.0038$); Figure 20C-D).

Because c-Rel$^{-/-}$ mice have previously been shown to exhibit defects in acute IL-12 responses to *T. gondii* at the site of infection (Mason et al., 2004a), it was pertinent to determine if this cytokine could restore the generation of the CD8^{+} T cell response. Therefore, c-Rel$^{-/-}$ mice were treated i.p. with exogenous IL-12 to determine if this cytokine was able to restore antigen-specific CD8^{+} T cell responses. In the PECs, this treatment resulted in increased frequencies and numbers of OVA-specific CD8^{+} T cells (Figure 21A, C). However, this treatment did not consistently restore CD8^{+} T cell responses in the spleen (Figure 21B, D). We also determined that c-Rel$^{-/-}$ mice given IL-12p70 at the time of immunization generated increased numbers and frequencies of antigen-specific IFN-γ-producing cells in the PECs (Figure 22A-B), while the number of cytokine-producing cells was not consistently rescued in the spleens of IL-12-treated mice (data not shown). We speculate that this partial rescue is due to inadequate levels of

Figure 21. Treatment with IL-12 partially restores tetramer$^+$ responses of c-Rel$^{-/-}$ mice

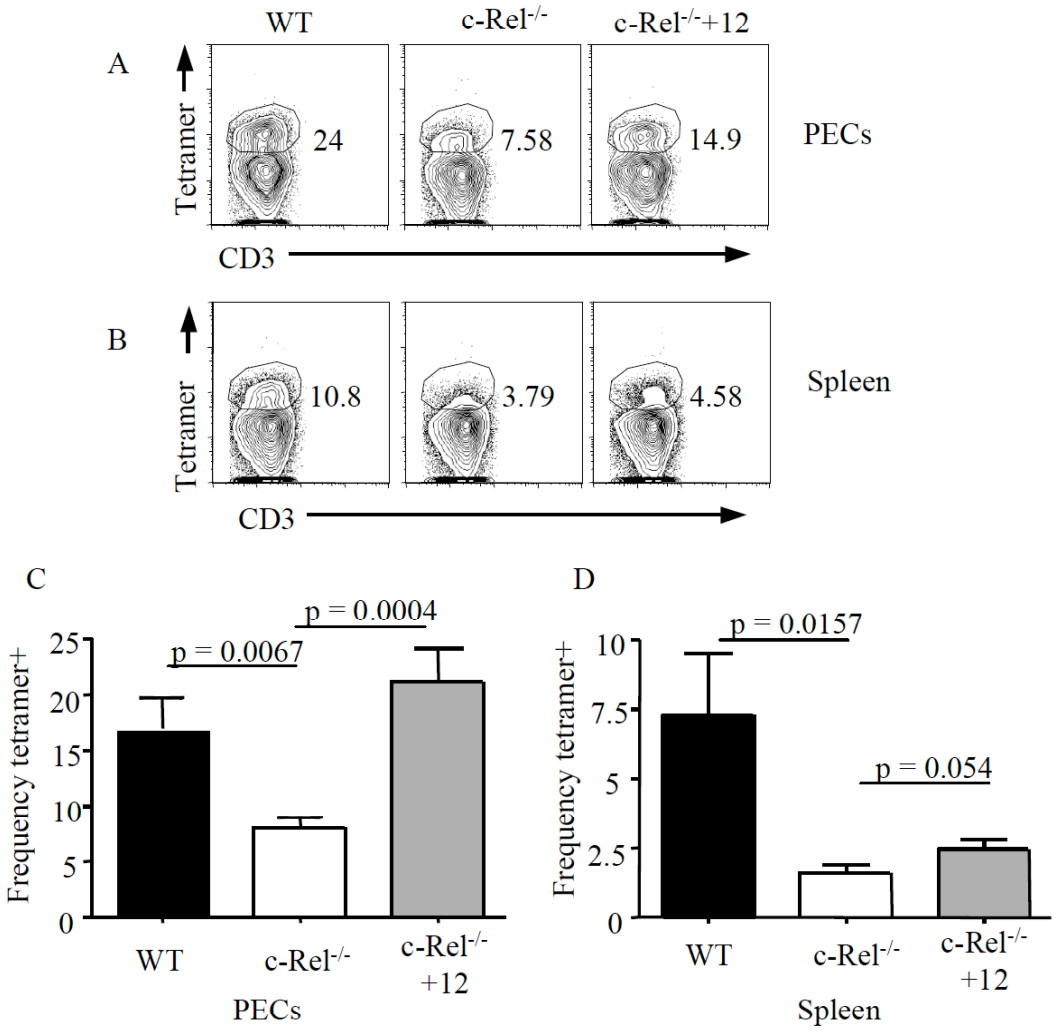


Figure 21. c-Rel$^{-/-}$ mice were treated with exogenous IL-12 and tetramer responses were analyzed 8 days following immunization with CPS-OVA. (A) Representative plots of tetramer responses from the PECs. (B) Representative plots of tetramer responses from the spleen. (C) Average + standard deviation for tetramer$^+$ frequency of PECs. (D) Average + standard deviation for tetramer$^+$ frequency of splenocytes.

Adapted in part from KA Jordan et al., International Immunology (under revision).

Figure 22. Treatment with IL-12 restores cytokine responses of c-Rel$^{-/-}$ PECs cells

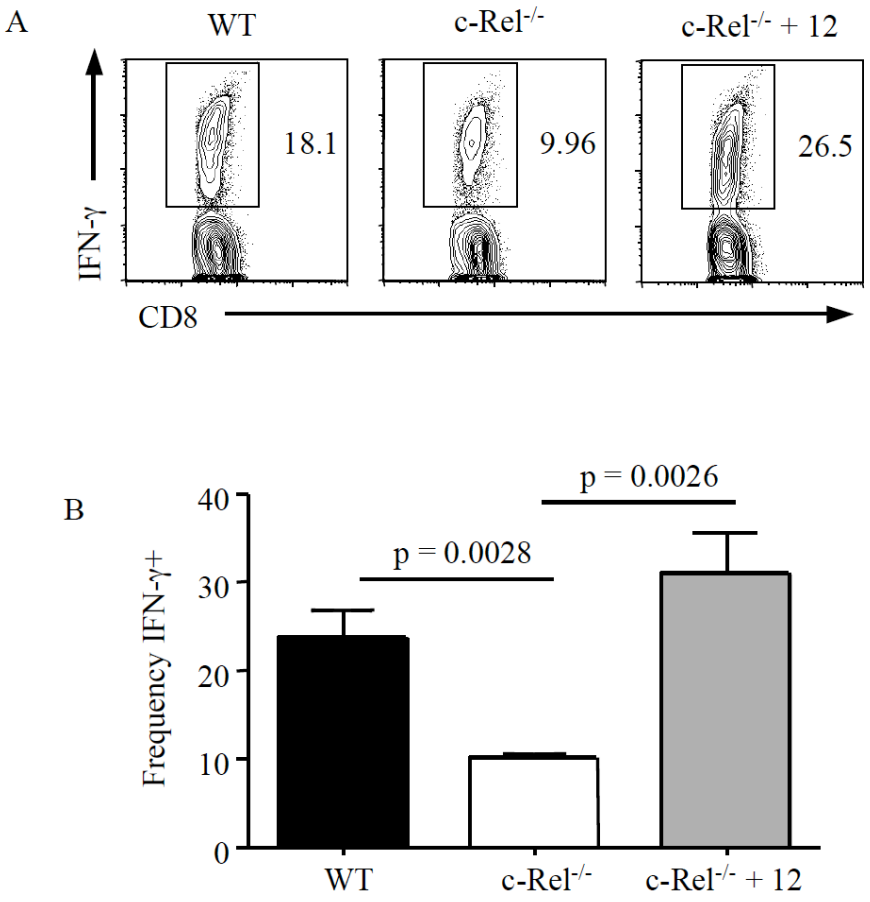


Figure 22. c-Rel mice were treated with IL-12 as in Figure 21. PECs were stimulated *ex vivo* with OVA peptide + BFA for 5 hours, after which cells were surface stained and fixed, then stained for intracellular cytokine production. (A) Representative plots, gated on CD8$^+$ T cells. Numbers represent frequency of IFN-γ + cells. (B) Average + standard deviation for IFN-γ+ cells from the PECs.

Adapted from KA Jordan et al., International Immunology (under revision).

the cytokine reaching the spleen, while ample levels were present in the site of injection. Alternatively, it is possible that another factor, such as failure of a required subset of cells to migrate into spleen, could also be responsible for the lack of a normal $CD8^+$ T cell response in the spleen of c-$Rel^{-/-}$ mice. Regardless, in the CPS-OVA immunization model, IL-12 dictates both the initial expansion, as well as the effector phenotype, of the antigen-specific $CD8^+$ T cell population.

4.6 c-Rel and the formation of memory $CD8^+$ T cells

Attempts to clarify the role of c-Rel in the generation or maintenance of $CD8^+$ T cell memory have been limited, but the approach of using replication-deficient parasites allowed for investigation of these responses at late time-points following infection. Thus, various tissues of mice were examined 2-3 months following immunization, and this analysis showed that not only were antigen-specific cells maintained in the spleen, but unexpectedly, they were present at levels similar to the WT controls (Figure 23A-B). Similar frequencies of tetramer-specific cells were also identified in the blood and bone marrow, indicating that c-$Rel^{-/-}$ mice were able to maintain a pool of memory $CD8^+$ T cells following immunization. It was not clear that antigen-specific cells induced by CPS-OVA would be able to survive, since c-Rel has been associated with upregulation of pro-survival family members. However when the expression of Bcl-2 was examined in WT and c-$Rel^{-/-}$ $tetramer^+$ splenocytes 35 days after immunization, there was no significant difference in terms of percentage (52.9 ± 3.45 vs. 52.6 ± 0.9) or MFI (318 ± 1.5 vs. 334.5 ± 3.5).

Although the frequency of memory $CD8^+$ T cells was comparable in WT and c-$Rel^{-/-}$ mice, additional studies were performed to compare their phenotype. At the time-points examined, up to 3 months post-immunization, antigen-specific $CD8^+$ T cells in both WT and c-$Rel^{-/-}$ mice were primarily $KLRG1^{high}$ and $CD62L^{low}$, therefore resembling effector cells. KLRG1, which was significantly lower on c-$Rel^{-/-}$ tetramer cells at acute time-points, was now expressed at a similar frequency on OVA-specific cells (data not shown). CD27, a member of the TNF receptor superfamily previously demonstrated to be expressed more highly on memory $CD8^+$ T cells, was expressed more highly on c-$Rel^{-/-}$ as compared to WT $tetramer^+$ cells (MFI 491 ± 9 vs. 589 ± 4.7; p = 0.0006). A significant difference was also noted in the expression of CD127, the IL-7Rα, which was also higher on c-$Rel^{-/-}$ $tetramer^+$ cells both in terms of frequency of IL-7Rα cells (Figure 23C; 67.7 ± 2.9 vs. 88.5 ± 0.8, $p<0.0001$) as well as MFI (236 ± 10.5 vs. 305 ± 15; p = 0.0028).

Another capacity correlated with memory $CD8^+$ T cell function is their ability to expand more rapidly compared to naïve $CD8^+$ T cells following pathogen exposure (Kaech et al., 2002). Because immunized c-$Rel^{-/-}$ mice were not able to survive rechallenge with RH-OVA, another approach was taken to assess the memory responses of antigen-specific $CD8^+$ T cells. WT and c-$Rel^{-/-}$ mice were immunized with 10^5 CPS-OVA parasites, and 30 days later naïve and immune mice were challenged with a ten-fold higher dose of CPS-OVA (10^6). 5 days following challenge, the expansion and function of the antigen-specific CD8+ T cell response was analyzed. As expected, there was an expansion of tetramer-specific cells in WT mice following challenge (Figure 24).

Figure 23. Memory cell phenotypes of WT and c-Rel$^{-/-}$ OVA-specific CD8^{+} T cells

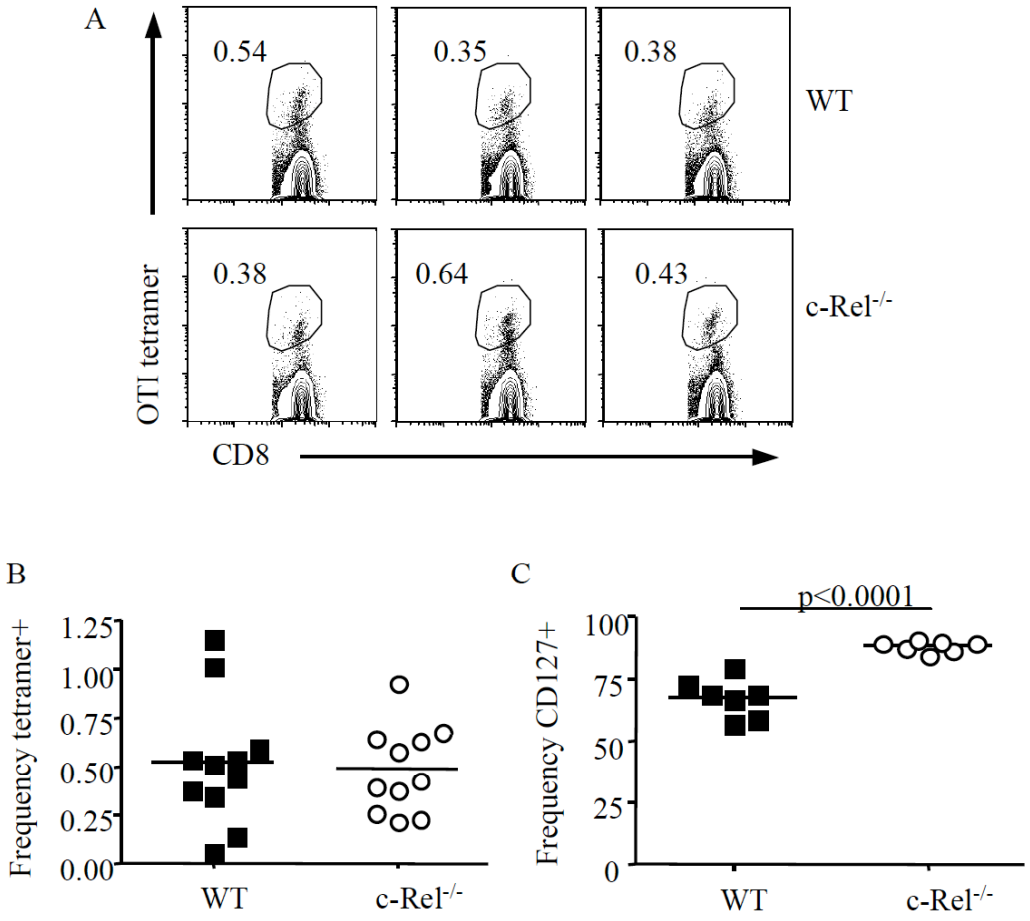


Figure 23. Spleens were analyzed 60-75 days following immunization for tetramer^{+} cells. (A) Representative dot plots, gated on CD3^{+}CD8^{+} cells, 75 days post-immunization. (B) Frequency of tetramer^{+} splenocytes, out of CD3^{+}CD8^{+} cells. (C) Frequency of tetramer^{+} cells that express high levels of CD127. For B and C, data are combined from multiple similar experiments, 60-75 days post-immunization.

Adapted from KA Jordan et al., International Immunology (under revision).

Figure 24. c-Rel is not required for CD8$^+$ T cell expansion following secondary challenge

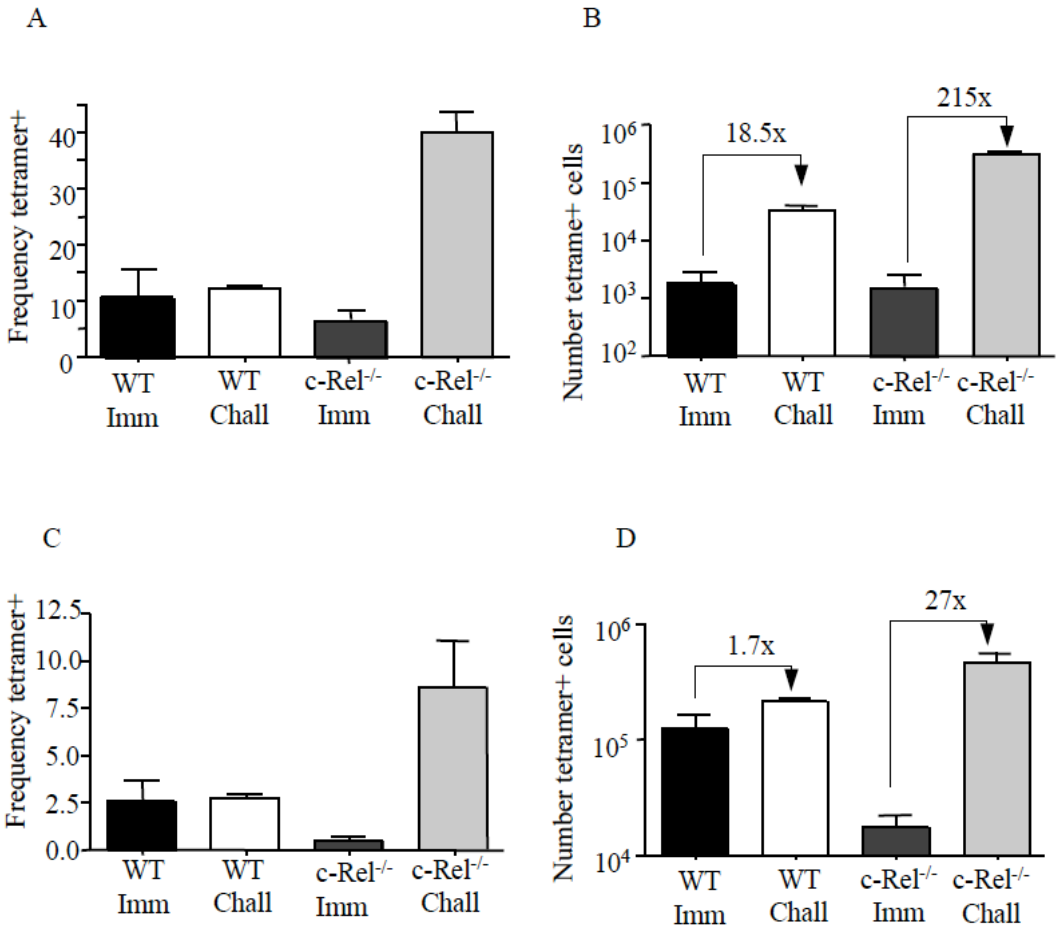


Figure 24. c-Rel is not required for secondary expansion following secondary challenge. Mice were immunized with 10^5 CPS-OVA, and 30 days later rechallenged with 10^6 CPS-OVA. Tetramer responses were then analyzed 5 days later. Frequency (A) and number (B) of tetramer$^+$ cells in the PECs. Frequency (C) and number (D) of cells in the spleen. Data from one of three similar experiments are shown.

Adapted from KA Jordan et al., International Immunology (under revision).

Figure 25. Secondary challenge following adoptive transfer

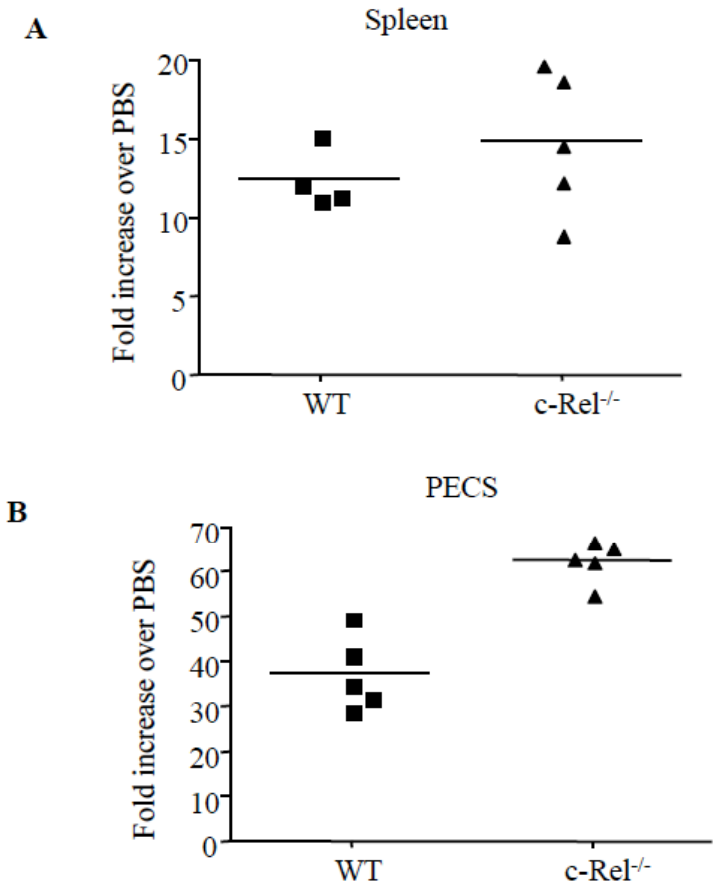


Figure 25. c-Rel is not required for secondary expansion following rechallenge. Mice were immunized with 10^5 CPS-OVA, and 30 days later T cells were isolated and approximately 10^7 cells were transferred to congenic recipients. Mice were challenged with 10^6 CPS-OVA and tetramer responses were analyzed 7 days later. Data from one of two similar experiments are shown, where fold increase of tetramer+ cells in the spleen (A) or PECs (B) was calculated by comparing CPS-OVA rechallenged mice to those given PBS following cell transfer.

Adapted from KA Jordan et al., International Immunology (under revision).

Figure 26. Cytokine responses of c-Rel$^{-/-}$ mice are rescued in a WT environment

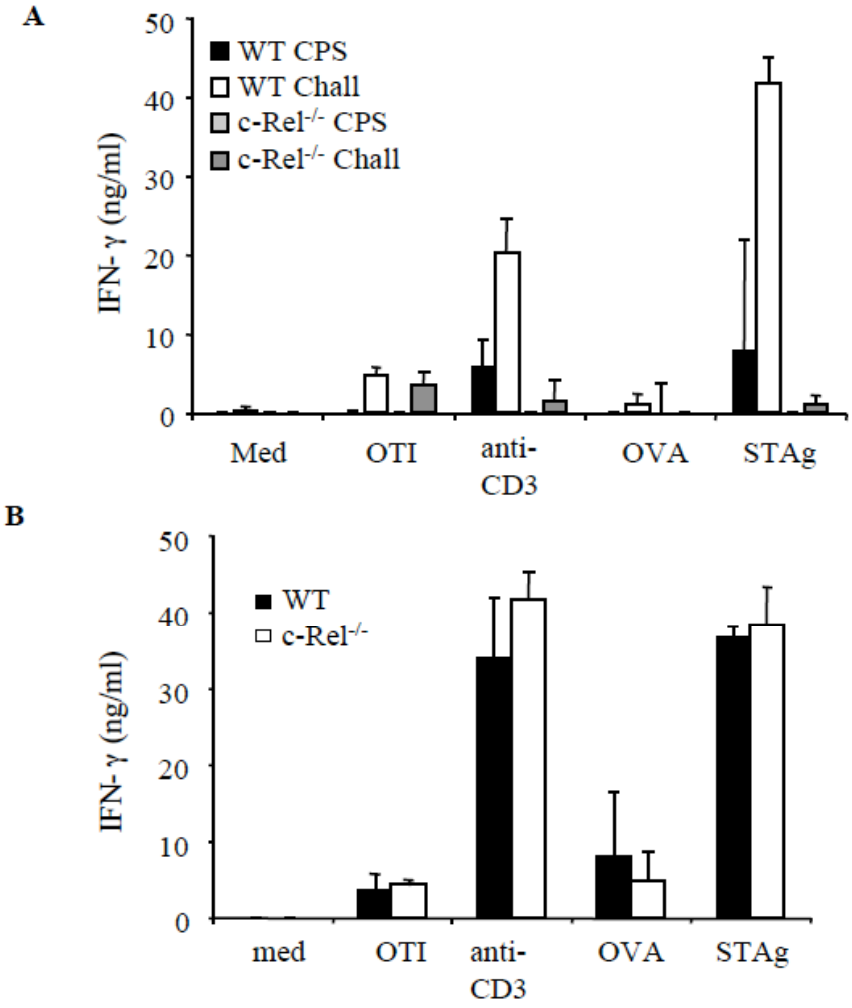


Figure 26. c-Rel$^{-/-}$ mice have impaired cytokine responses that can be rescued in a WT environment. (A) Cytokine responses of immunized (CPS; day 35 post-immunization) or immunized and challenged (Chall; day 5 post-challenge) WT and c-Rel$^{-/-}$ splenocytes were assessed by ELISA. Data represent average ± standard deviation with 3-4 mice per group from one of two similar experiments.(B) Cytokine responses from splenocytes as described in Figure 24 were measured by ELISA. Data represent average ± standard deviation for 5 mice per group from one representative experiment.

Adapted from KA Jordan et al., International Immunology (under revision).

However, despite the requirement for c-Rel in the induction of the primary $CD8^+$ T cell response, OVA-specific cells from the PECs or spleen of c-$Rel^{-/-}$ mice expanded to a much greater degree. Closer examination revealed that the increase in frequency of $tetramer^+$ cells was significantly higher in the PECs ($p = 0.001$) as well as the spleen ($p = 0.29$) of challenged c-$Rel^{-/-}$ mice compared to the challenged WT mice, when each group was compared to mice that had been immunized 30 days prior (Figure 24A, C). Similarly, the increase in number of $tetramer^+$ cells was significantly higher in the spleen ($p = 0.01$) as well as the PECs ($p = 0.0008$) of challenged c-$Rel^{-/-}$ mice, compared to challenged WT mice (Figure 24B, D). These results were confirmed in experiments in which T cells were isolated from immunized WT and c-$Rel^{-/-}$ mice, and transferred to naïve mice that were then challenged with 10^6 CPS- OVA. Analysis of the tetramer-specific population revealed that c-$Rel^{-/-}$ $CD8^+$ T cells maintained their enhanced ability to proliferate following rechallenge (Figure 25A-B), with larger differences noted in the PECs (fold increase of 37.4 ± 3.7 for WT vs. 63.4 ± 2.3, $p = 0.0004$) as compared to the spleen (fold increase of 12.45 ± 0.9 for WT vs. 14.83 ± 2, p = n.s.).

Despite this ability to greatly expand the $CD8^+$ T cell population, c-$Rel^{-/-}$ mice were unable to survive secondary lethal challenge, as shown in Figure 13. Therefore experiments were performed to assess whether the effector function of c-$Rel^{-/-}$ memory $CD8^+$ T cells was altered. As shown in Figure 26, while cells from immune or challenged WT mice produced IFN-γ in response to most of the stimuli, splenocytes from c-$Rel^{-/-}$ mice had an impaired ability to produce IFN-γ following stimulation with OVA protein, STAG, or anti-CD3, and in fact made very low levels of cytokine in response to OVA

peptide (Figure 26A). In contrast, when immune c-Rel$^{-/-}$ T cells were adoptively transferred in WT mice and then rechallenged with CPS-OVA, the WT environment restored their ability to make IFN-γ (Figure 26B). Thus, one potential explanation for the inability of c-Rel$^{-/-}$ mice to survive lethal rechallenge is their inability to produce sufficient levels of cytokines to protein antigens. One caveat of this adoptive transfer set-up is that the challenge dose of CPS-OVA utilized in this experiment induced an endogenous host response easily identified by tetramer staining; thus, the ELISA responses could be attributed at least in part to the host response. A more thorough analysis of donor cell cytokine production would be needed to confirm that the c-Rel$^{-/-}$ CD8^{+} T cell responses were effectively rescued by the WT host environment, and in the future this could be assessed ex vivo by flow cytometry to examine donor-specific cytokine responses.

4.7 Conclusions

Previous studies demonstrated the importance of the transcription factor c-Rel in the development of a potent Th1 immune response to the parasite *T. gondii* and have also implicated c-Rel in the development of T cell responses in a variety of other settings (Campbell et al., 2000; Hilliard et al., 2002; Mason et al., 2004b; Wang et al., 2008). In contrast, another report has indicated that c-Rel was not required for the activation and effector function of CD8^{+} T cells in the context of influenza infection (Harling-McNabb et al., 1999). Attempts to assess the role of c-Rel in the generation of CD8^{+} T cell responses to toxoplasmosis have been hindered by the susceptibility of c-Rel$^{-/-}$ mice to this organism. This difficulty was overcome by utilizing a non-replicating strain of *T.*

gondii engineered to secrete the model antigen OVA, thus allowing the primary and secondary antigen specific $CD8^+$ T cell response to be tracked with improved specificity (Jordan et al., 2009). Unexpectedly, use of this experimental system revealed that while c-Rel is required for optimal generation of $CD8^+$ T cells in response to *T. gondii*, it is not intrinsically needed by $CD8^+$ T cells for their expansion or effector function.

There are several possible explanations for the role of c-Rel in the development of an optimal primary $CD8^+$ T cell response. IL-12 is essential for protective $CD8^+$ T cell responses to *T. gondii* and previous studies have identified a key role for c-Rel in the production of IL-12 (Wilson et al., 2008). Although there are also c-Rel-independent pathways for IL-12 production (Mason et al., 2002) there is a defect in early IL-12 in c-$Rel^{-/-}$ mice infected with *T. gondii* (Mason et al., 2004a). Moreover, studies by Gerondakis and colleagues highlighted a role for c-Rel in regulating the differentiation and survival programs of plasmacytoid dendritic cells (O'Keeffe et al., 2005), which are an important source of IL-12 during toxoplasmosis. Additionally, recent work has associated the expression of KLRG1 by $CD8^+$ T cells with exposure to IL-12 (Wilson et al., 2008), and while antigen-specific $CD8^+$ T cells from c-$Rel^{-/-}$ mice were for the most part phenotypically similar to WT antigen-specific $CD8^+$ T cells, the only major difference was reduced expression of KLRG1. In addition to regulating the effector phenotype of $CD8^+$ T cells, IL-12 also impacts the initial proliferation of these cells, which likely explains the impaired expansion of $CD8^+$ T cells in c-$Rel^{-/-}$ mice. Indeed, providing exogenous IL-12 to c-$Rel^{-/-}$ mice resulted in a partial rescue of $CD8^+$ effector T cell expansion following immunization.

The observation that OVA-specific $CD8^+$ T cells could expand normally when transferred to WT recipients and challenged further supports the idea that c-Rel is playing a $CD8^+$ T cell-extrinsic role during *T. gondii*. For example, several groups have reported reduced ability of c-$Rel^{-/-}$ $CD4^+$ T cells to produce IL-2, and the ability of the CPS-OVA parasites to promote $CD8^+$ T cell responses is dependent on $CD4^+$ T cells. Thus, defects in the $CD4^+$ T cell compartment, in terms of numbers or in terms of functionality, would also impact the $CD8^+$ T cell response. Indeed, while the ability of c-$Rel^{-/-}$ OTI cells transferred into WT mice to expand normally indicates that c-Rel is not intrinsically required for optimal $CD8^+$ T cell responses, these experiments do not distinguish between the role of c-Rel in antigen-presenting cell populations and $CD4^+$ T cell help.

Perhaps the most surprising element of these studies was that although c-$Rel^{-/-}$ mice failed to survive a secondary challenge with virulent *T. gondii*, there were comparable levels of memory $CD8^+$ T cells in the WT and c-$Rel^{-/-}$ mice. Further, upon rechallenge c-$Rel^{-/-}$ mice exhibited increased expansion of the $CD8^+$ T cell subset compared to WT mice. This phenotype may also be a function of reduced production of IL-12 as several studies indicate that although IL-12 is important for $CD8^+$ effector T cell generation and function, its absence allows $CD8^+$ T cells to form better memory (Joshi et al., 2007; Pearce and Shen, 2007). This conclusion seems to depend on the system used, as IL-12 has not been shown to induce similar effects in all infection models. IL-12 also seems to be important in the expansion of effector $CD8^+$ T cells in response to *L. monocytogenes* and *T. gondii* (Pearce and Shen, 2007; Wilson et al., 2008). However, IL-12 is not as critical in some viral systems including LCMV and VSV, while there are

contradictory reports about its role in $CD8^+$ T cell responses against vaccinia virus (Keppler et al., 2009; Thompson et al., 2006).

Several viral and bacterial systems have been used extensively to define the factors that influence the development of $CD8^+$ T cell effector and memory populations. Perhaps not surprisingly, in other infectious settings, there are some differences emerging; for instance the acquisition of a $CD62L^+$ memory phenotype seems to take longer in parasite infection as compared to LCMV (Bustamante et al., 2008; Wherry and Ahmed, 2004). Certainly, much work remains to be done in the study of memory $CD8^+$ T cells that develop in distinct inflammatory environments and the contributions made by different cytokines, including IL-12. However, our results are in agreement with a model supporting a role for IL-12 in the promotion of primary responses while its absence enhances development of memory $CD8^+$ T cells, and this will be further discussed in Chapter 5. Determining the factors that regulate $CD8^+$ T cell effector and memory development has strong implications for rational vaccine design, or control of immune system dysfunction. Overall, it seems likely that c-Rel is not required for the function of mature $CD8^+$ T cells. This is in agreement with previous work that showed relatively normal function and proliferation of effector $CD8^+$ T cells induced following infection of c-$Rel^{-/-}$ mice with influenza and HSV (Harling-McNabb et al., 1999; Mintern et al., 2002). Rather, the work presented here suggests that c-Rel plays a significant role in creating the inflammatory environment that influences the development and function of the $CD8^+$ T cell response and memory formation.

Chapter 5: Discussion and Future Directions

5.1 Introduction

As introduced earlier in this thesis, *T. gondii* is a parasite of major human and veterinary importance. It has also provided an excellent model to help define many of the general principles that define resistance to other parasitic infections, and provided insights into the innate mechanisms of resistance to intracellular organisms and how adaptive immunity functions during chronic infection. While a number of immune cell subsets contribute to resistance against this pathogen, $CD8^+$ T cells that produce the cytokine IFN-γ are a critical component of the immune response against *T. gondii*, and are the focus of this thesis. While the protective capacity of this immune cell subset had been known for some time, gaps in knowledge remained, particularly as related to the factors that contribute to the generation of protective $CD8^+$ T cell responses. The beginning stages of this thesis were dedicated to understanding the cellular immune response to immunization, utilizing a strain of the parasite that was unable to replicate in mice. These parasites allowed us to study memory responses even in mice that are normally susceptible to acute infection. This work revealed a requirement for $CD4^+$ T cell help in the $CD8^+$ T cell effector response to immunization. The latter part of this thesis focused on using this model to understand how one transcription factor from the NF-κB family, c-Rel, could influence $CD8^+$ T cell responses to *T. gondii*. These studies showed that despite being implicated in T cell proliferation and survival, c-Rel was not intrinsically required for $CD8^+$ T cell expansion in response to *T. gondii*, nor was it

required for secondary expansion. Rather, the sub-optimal inflammatory environment, associated with reduced production of IL-12, contributed to shaping the size and phenotype of the $CD8^+$ T cell response. The importance and context of these findings will now be discussed.

5.2 The inflammatory environment

It has long been appreciated that the environment in which T cells are generated contributes to their activation and effector function; for example, different pathogens are known to induce a diverse set of outcomes, causing $CD4^+$ T cells to differentiate either into Th1 or Th2 cells, characterized by different patterns of cytokine production (Murphy and Reiner, 2002). The inflammatory environment, including infected or activated antigen-presenting cells and cytokines and chemokines they produce, contributes to this differentiation process of $CD4^+$ T cells (Murphy et al., 2000). More recently, a more nuanced approach has made clear that the inflammatory environment can also contribute to the differentiation of $CD8^+$ T cells by impacting either the size or the phenotype of the population through, for example, IL-12 or Type I interferons (Joshi et al., 2007; Pearce and Shen, 2007; Thompson et al., 2006). During *T. gondii* infection, as for many organisms, differences in inflammation will be a function of multiple variables including type and dose of infection, parasite virulence factors, and numerous host factors.

The contribution of the inflammatory environment to $CD8^+$ T cell development during *T. gondii* infection is complex because there are three major clonal strains of this parasite that possess disparate abilities to replicate, disseminate and cause disease *in vivo*. Virulent strains of *T. gondii* demonstrate widespread parasite dissemination and tissue

destruction, resulting in systemically high levels of IL-12 and IFN-γ that have been linked to host pathology (Gavrilescu and Denkers, 2001). Virulence factors have recently been identified and differ between strains, because expression of specific rhoptry proteins from Type I strains could render Type II or Type III strains of *T. gondii* pathogenic to mice (Saeij et al., 2006; Taylor et al., 2006). All of these strains induce a lytic infection, and this parasite destruction of tissues upon host cell egress also contributes to the inflammatory environment (Black and Boothroyd, 2000).

It has been known for some time that *T. gondii* can dampen the pro-inflammatory response through interference with multiple intracellular signaling pathways (Denkers, 2003). For instance, several groups have found that *T. gondii* interferes with NF-κB at the level of subunit phosphorylation (Shapira et al., 2002) and nuclear translocation of this transcription factor (Butcher and Denkers, 2002; Butcher et al., 2001). *T. gondii* also induces the anti-inflammatory cytokine IL-10, which through its direct inhibition of IL-12 can dampen developing immune responses against the parasite (Butcher et al., 2005b; Khan et al., 1995). Additionally, some work has suggested that more virulent strains of *T. gondii* have enhanced migration, which could contribute to dissemination and virulence (Barragan and Sibley, 2002). Thus, the complicated life cycle of *T. gondii* impacts the inflammatory environment in infected hosts by expressing virulence factors, or by directly inhibiting host immune responses.

In other models of infectious disease, antigen burden is another factor that influences the developing $CD8^+$ T cell response. For instance, during *L. monocytogenes* infection, using antibiotic treatment to limit antigen exposure regulates the size of the

$CD8^+$ effector T cell pool in some studies (Mercado et al., 2000) but not others (Williams and Bevan, 2004). To limit the number of variables that would affect $CD8^+$ T cell development, I took advantage of CPS-OVA parasites that provided a system of limited antigen exposure and host cell cytolysis. Though CPS-OVA was derived from a Type I strain, I would expect limited production of any virulence factors produced after immunization due to the lack of parasite replication and subsequent parasite dissemination. The availability of different strains that expressed OVA allowed the comparison of immune responses that developed in environments with varying levels of inflammation. I found that 8 days following infection, the frequency of OVA-specific cells in the $CD8^+$ T cell population inversely correlated with the amount of inflammation. Thus, the replication-deficient CPS-OVA parasites induced the highest levels of antigen-specific $CD8^+$ T cells, followed by the Pru-OVA Type II strain parasites (Chapter 3), while the highly virulent RH-OVA Type I strain generated barely detectable levels of OVA-specific $CD8^+$ T cells (Tait et al., 2010).

The studies presented in this thesis and demonstrated by Tait and colleagues also correlated the induction of a *T. gondii*-specific $CD8^+$ T cell population in response to Pru-OVA or RH-OVA with the proliferation or recruitment of $CD11c^+$ and myeloid DC populations to the site of infection. However, while a $CD11b^+CD8^-$ DC population did expand following Pru-OVA infection, the DC populations seen in the PECs following CPS-OVA closely resembled those from naïve mice (Figure 27A). Additionally, while Pru-OVA induced upregulation of MHC Class II, CD80 and CD86, the phenotype of DCs identified during CPS-OVA more closely resembled naïve mice (Figure 27B and data not shown).

Figure 27. CPS-OVA induces lower levels of DC activation compared to Pru-OVA infection

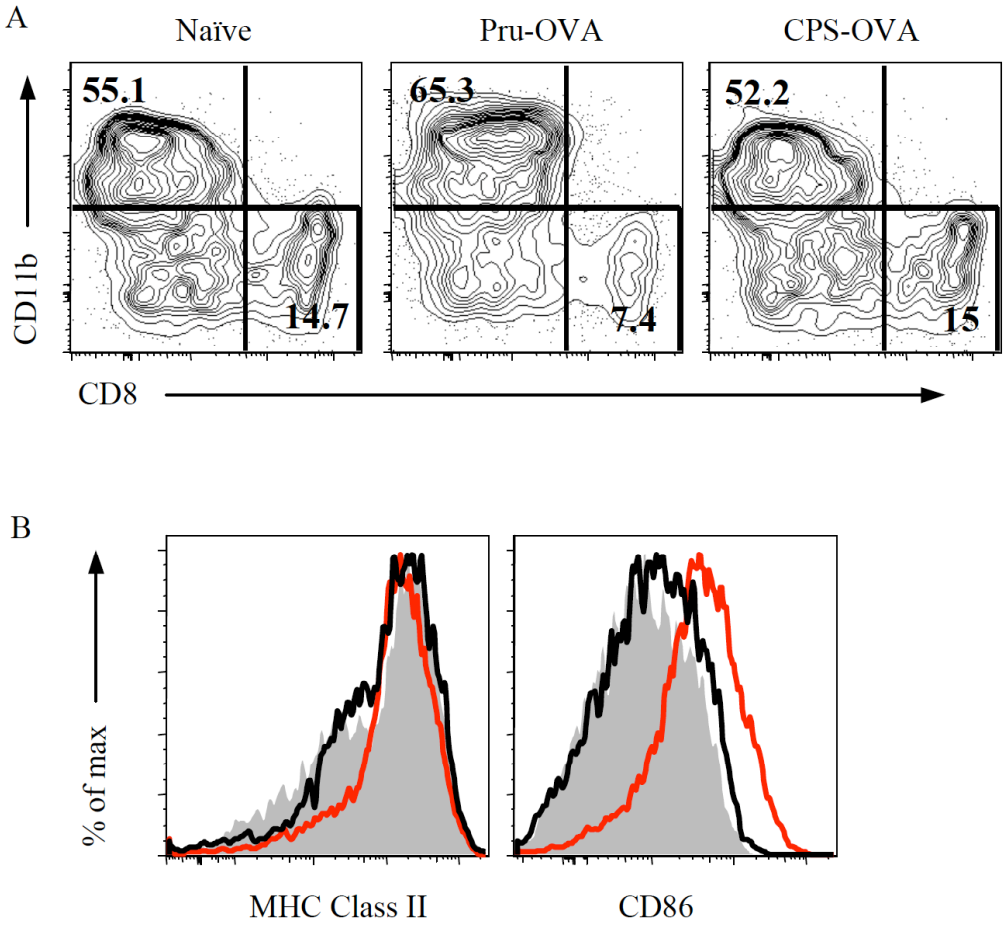


Figure 27. Mice were infected with 10^5 CPS-OVA or Pru-OVA and splenic DC were analyzed 8 days post-infection. (A) While Pru-OVA induces changes in the population structure of splenic DC, CPS-OVA DC (gated on CD11c+ cells) resemble DC from naïve mice. (B) Expression of activation markers on splenic DC from naïve mice (gray shaded histogram), as compared to mice infected with Pru-OVA (red line) or CPS-OVA (black line). This experiment was done once with 3 mice per group.

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Thus, the presence of expanded and activated DC populations, at least at the time points we examined, was not required for CPS-OVA parasites to induce a potent $CD8^+$ T cell response (as demonstrated in Chapter 3.3). Because we found no differences in DC populations that might explain the ability of CPS-OVA to induce this robust OVA-specific $CD8^+$ T cell population, I next wanted to examine more specific factors known to contribute to effector cell generation during *T. gondii* infection.

Numerous studies have examined the importance of dendritic cell production of IL-12 and priming of $CD8^+$ T cell responses during *T. gondii*, and the depletion as well as rescue experiments discussed earlier in this thesis clearly demonstrate a role for this cytokine during infection with CPS-OVA. While other groups have reported that CPS parasites induce high systemic levels of IL-12p70 (Gigley et al., 2009), we have been unable to confirm their findings. Thus, despite the observation that CPS parasites induce no overt inflammation, do not disseminate widely, and cannot cause cytolysis of host cells, they do generate protective responses to rechallenge. Further, the generation of an OVA-specific $CD8^+$ T cell response to immunization was dependent on IL-12 and $CD4^+$ T cell help. While previous work in other infections suggests a model where the ability of a pathogen to generate a robust $CD8^+$ T cell depends on the degree of inflammation and the amount of antigen exposure, the CPS-OVA experiments presented here suggest that "more" might not always equal "better" in terms of the generation of protective $CD8^+$ T cell responses.

5.3 Antigen presentation

Naïve $CD8^+$ T cells are activated by professional antigen-presenting cells that present the correct peptide in the context of MHC Class I on their cell surface. It was previously believed that only cell-associated endogenous antigens were presented by MHC Class I molecules, consistent with models in which $CD8^+$ T cells can only recognized virally-infected cells or cancer cells. More recently, it has been appreciated that during transplant rejection, or during infections that target parenchymal cells, professional APC can sample and display exogenously-derived proteins on MHC I in the process of cross-presentation (Rock and Shen, 2005). A model of transient dendritic cell (DC) depletion was important in clarifying that this pathway of cross-priming was critically required for generation of antigen-specific $CD8^+$ T cells in mice infected with *L. monocytogenes* or *P. yoelii* (Jung et al., 2002; Liu et al., 2006). However, it must be noted that there is evidence in favor (John et al., 2009), as well as evidence against (Dzierszinski et al., 2007; Goldszmid et al., 2009; Gubbels et al., 2005), cross-presentation of antigen to $CD8^+$ T cells during toxoplasmosis. Distinguishing which pathways are involved in these events may not just be of academic interest as they impact the type of pathogen antigens that are presented and may likely influence generation vaccine-induced adaptive immunity. As will be discussed in the next section, model antigen systems have been a key component to deciphering the pathways of antigen presentation during parasite infection.

5.3.1 Model antigen systems

At the time these thesis studies were initiated, the lack of defined endogenous *T. gondii* $CD8^+$ T cell epitopes meant that the ability to study antigen-specific T cell responses was restricted. Similar issues have been encountered in many infectious systems and with the advent of TCR transgenic mice, one strategy that has been employed is the use of model antigens (Bertholet et al., 2006; Garg et al., 1997; Guzman et al., 1998; Pan et al., 1995). Accordingly, a Type II strain of *T. gondii* expressing the model antigen β-galactosidase was engineered and the response to this surrogate microbial antigen was used to examine the immune response (Kwok et al., 2003; Lutjen et al., 2006). A similar approach was initiated in the Hunter laboratory utilizing the model antigen ovalbumin (Pepper et al., 2004). One strength of the OVA model system is the extensive set of tools that has been generated: TCR transgenic mice, tetramers to track antigen-specific cell populations, and a T cell hybridoma useful for studying antigen presentation.

One caveat is that use of these transgenic systems may not accurately reflect what occurs during a polyclonal response, and until similar experiments can be conducted using endogenous *T. gondii* antigens these findings must be interpreted with care. Nevertheless, recent work has described two *T. gondii* endogenous $CD8^+$ T cell epitopes in BALB/c mice, one derived from the dense granule protein GRA4, and the other from the rhoptry protein ROP7 (Frickel et al., 2008). The Shastri laboratory identified an immunodominant $CD8^+$ T cell epitope derived from GRA6, another secreted protein (Blanchard et al., 2008). As was demonstrated earlier with parasites that expressed model antigens, the endogenous epitopes were located in secreted but not cytosolic proteins,

serving as a confirmation of our model antigen approach. However, the fact that only three endogenous epitopes have been identified from the many thousands of *T. gondii* genes illustrates some of the difficulties associated with antigen presentation and epitope discovery in parasites. One caveat is that the model antigens expressed by our transgenic parasites might be secreted at higher levels than the endogenous proteins identified by Frickel and colleagues, and experiments are underway to determine the amount of ovalbumin that is secreted by different strains of *T. gondii*. Differences in amount of protein secreted could explain why the levels of antigen-specific cells we identify by tetramer staining during Pru-OVA or CPS-OVA infection are higher than what has been seen with these endogenous epitopes (Blanchard et al., 2008; Frickel et al., 2008).

These model antigen systems have also helped to clarify how antigen presentation occurs during *T. gondii*. Earlier work from the Roos and Hunter laboratories has provided two different perspectives on whether or not only infected cells are able to present antigen (Dzierszinski et al., 2007; John et al., 2009). It is likely that antigen presentation pathways differ between Pru-OVA and CPS-OVA. As already mentioned, CPS-OVA induces an immune response without host cell lysis, thus indicating that direct presentation of antigen by infected cells is sufficient for priming of $CD8^+$ T cell responses during CPS-OVA. The use of two-photon microscopy to visualize the interactions between antigen-specific T cells and DC during the priming phase is already yielding critical insight into successful T cell priming, and this technique has already been used by the Hunter laboratory and others to examine the behavior of different cell populations during infection (John et al., 2009; Peters et al., 2008; Schaeffer et al., 2009; Tarun et al., 2006; Wilson et al., 2009). However, this comparison is technically

challenging, not least because of caveats due to using large numbers of adoptively transferred cells, because the transfer of high numbers of TCR transgenic cells can alter the kinetics, expansion, and phenotype of the immune response (Badovinac et al., 2007; Badovinac and Harty, 2007). Despite this fact, two-photon microscopy promises to reveal new insights into innate responses to infection and the interactions between different cell populations *in vivo*.

5.4 c-Rel in Antigen Presentation

The use of model antigens as described above provided advantageous tools for studying antigen presentation, since defined Class I- and Class-II restricted epitopes within the OVA protein induce an endogenous T cell response. While IL-12 levels did enhance the size of the effector $CD8^+$ T cell population, another potential influence on T cell expansion could be that c-Rel is required for optimal antigen presentation. In a model of influenza infection, c-$Rel^{-/-}$ mice were able to mount a CTL response and had a small delay in viral clearance, but this group did not determine if the flu-specific $CD8^+$ T cell population itself was reduced or less functional than WT mice (Harling-McNabb et al., 1999). Another group found that c-Rel was not required for DC maturation or expression of CD86 following LPS stimulation but that c-$Rel^{-/-}$ DCs were defective at inducing allogeneic T cell stimulation (Boffa et al., 2003). Additionally, c-Rel was previously shown to be required for the generation of $CD8^+$ T cell responses in a model of immunization dependent on cross-presentation, while it was not required to induce $CD8^+$ T cell responses to herpes simplex virus (Mintern et al., 2002). Because IL-12 could rescue the antigen-specific $CD8^+$ T cell population in c-$Rel^{-/-}$ mice, this implies there was

no impairment in antigen processing and presentation, though this needs to be studied more in-depth.

An additional implication would be that either that c-Rel is not required for cross-presentation in the CPS-OVA model, or that this model does not require cross-priming to induce a $CD8^+$ T cell response. My work showed that $CD8^+$ T cells from c-$Rel^{-/-}$ mice had impaired cytokine responses to OVA protein but could respond similarly to WT cells when stimulated with OVA peptide, suggesting that there were issues with antigen processing or presentation in c-$Rel^{-/-}$ mice. Additionally, adoptive transfer experiments revealed that WT OTI-GFP cells had defective cytokine responses against OVA protein but not OVA peptide when transferred into c-$Rel^{-/-}$ mice, suggesting a defect in DC antigen presentation or processing. It would be interesting to look more closely at different components of antigen processing pathways in APCs because it is not known whether or not c-Rel directly targets any of these genes.

To further address the issue of antigen presentation, splenic $CD11c^+$ dendritic cells were sorted from infected WT and c-$Rel^{-/-}$ mice to see if they could present antigen *in vitro* to the B3Z cell line, a T cell hybridoma specific for ovalbumin. In these experiments I found no defect in the ability of c-$Rel^{-/-}$ splenic dendritic cells to activate B3Z cells, whether exogenous antigen was added or not, and if anything the c-$Rel^{-/-}$ DC induced more activation of the B3Z cells (WT OD 0.159 vs. c-Rel OD 0.221 for 10^5 DC without exogenous antigen; WT OD 1.48 vs. c-Rel OD 1.68 for 10^5 DC with exogenous OVA peptide). However, this cell line is known to have lower thresholds for activation or cytokine production as compared to naïve $CD8^+$ T cells and thus the ability of c-$Rel^{-/-}$ DC

to activate naïve T cells during *T. gondii* may be impaired. Related to this point, previous work from the Hunter laboratory has implicated one subset of DC, the plasmacytoid DC (pDC), in priming $CD4^+$ T cell responses to *T. gondii* (Pepper et al., 2008). Whether this subset contributes to $CD8^+$ T cell priming is unclear. Previous reports have demonstrated a two-fold reduction in the pDC population in c-$Rel^{-/-}$ mice, and demonstrated diminished IL-12 production by pDC in response to TLR9 stimulation (O'Keeffe et al., 2005). Therefore, it remains a possibility that defects in this population could contribute to the diminished $CD8^+$ T cell responses in c-$Rel^{-/-}$ mice though this needs to be examined in more detail.

5.5 c-Rel in T cells

This thesis work aimed not only at deciphering $CD8^+$ T cell responses to *T. gondii*, but was also concerned with the role played by the NF-κB transcription factor c-Rel. As c-Rel has previously been described as playing a critical role in T cell proliferation and cytokine production (Gerondakis et al., 1996; Liou et al., 1999), the initial hypothesis at the start of these studies was that c-Rel would be intrinsically required within the $CD8^+$ T cell population for their expansion, activation and effector function within the context of *T. gondii* infection. During LCMV infection, priming of $CD8^+$ T cells is dependent on Type I interferons (Thompson et al., 2006), however c-Rel is not required for upregulation of virus-induced Type I interferons (Wang et al., 2007), suggesting c-$Rel^{-/-}$ mice might experience different outcomes in terms of $CD8^+$ T cell responses depending on the inflammatory environment. Surprisingly, the data presented here demonstrated that c-Rel is not intrinsically required for the effector response of

$CD8^+$ T cells to CPS-OVA, and that in the absence of c-Rel secondary responses were enhanced. While c-Rel does not seem to be playing a T cell-intrinsic role in the CPS-OVA model system, it is clearly contributing to the generation of T cell responses through its role in APC and pro-inflammatory cytokines.

Indeed, one role that has been ascribed to c-Rel is that it functions to link innate and adaptive immunity, by making naïve but not effector T cells that receive pro-inflammatory cytokine signals more receptive to TCR triggering (Banerjee et al., 2005). As introduced earlier, study of individual NF-κB family members is complicated by the fact that they may serve overlapping roles in certain situations. This might be why it has been suggested that c-Rel modulates, but is not essential for, gene expression related to T cell activation (Bunting et al., 2007). This might differ depending on whether they are naïve, effector or memory $CD8^+$ T cells, because while naïve T cells are highly dependent on TCR signaling, memory cells can respond to cytokine signals alone, which would act through distinct signaling intermediates. It is also possible that c-Rel interacts with distinct molecules in $CD8^+$ T cells compared to other cell populations. For instance, c-Rel has been implicated as a key mediator of PKC-θ-mediated survival signals downstream of anti-CD3/anti-CD28 stimulation in $CD8^+$ but not $CD4^+$ T cells *in vitro* (Saibil et al., 2007).

Another potential explanation for the enhanced secondary expansion of c-$Rel^{-/-}$ $CD8^+$ T cells following challenge is the absence of thymically-derived regulatory T cells that has recently been demonstrated in c-$Rel^{-/-}$ mice (Isomura et al., 2009; Long et al., 2009; Ruan et al., 2009). Regulatory T cells are known to be an important factor

regulating the expansion of memory $CD8^+$ T cells (Kursar et al., 2002; Suvas et al., 2003), and at first we thought the absence of regulatory T cells might explain why the challenged c-$Rel^{-/-}$ mice had such a large expansion of OVA-specific $CD8^+$ T cells compared to the WT mice. However, the finding in Figure 24 indicates this might not be the case, because c-$Rel^{-/-}$ OVA-specific $CD8^+$ T cells adoptively transferred into WT hosts (that had normal numbers of T-regs) still expanded more than the WT memory cells following secondary challenge. The influence of regulatory T cells in this system could be more formally tested, for example by adoptive transfer of WT purified regulatory T cells into immune c-$Rel^{-/-}$ mice prior to secondary challenge. In any case, this would not fully explain the phenotype, because the c-$Rel^{-/-}$ OVA-specific cells were still able to expand more robustly than WT cells after adoptive transfer in WT recipients. One other possibility that we have considered is that c-Rel might be playing a negative regulatory role in the proliferation of memory $CD8^+$ T cells. This is, of course, completely contradictory to its role as previously described in many different models of infection. However, there is a precedent for NF-κB family members serving as negative regulators, because $p50^{-/-}$ natural killer (NK) cells proliferate better than WT NK cells, potentially via increased STAT5 activation (Tato et al., 2006). Further, other work has shown that p50 homodimers can act as inhibitors of gene expression to suppress the immune response (Carmody et al., 2007b; Zhong et al., 2002).

5.6 Memory during parasite infection

$CD8^+$ T cell memory has been studied extensively in a number of infectious models, and can be defined as the ability of antigen-specific cells to survive and provide

protective immunity long-term (Rocha and Tanchot, 2006). In recent years the LCMV system has provided a powerful experimental tool infection with a well-described primary infection that has been used to define $CD8^+$ T cell memory, with a peak in the viral load around day 3 and clearance of virus from the tissues by around day 8 (Harrington et al., 2000; Wherry et al., 2003). This, along with earlier work looking at human T cells (Sallusto et al., 1999), has lead to a model whereby 2 distinct subsets of memory $CD8^+$ T cells, central vs. effector, can be defined based on their anatomical location, cell surface molecules, and effector functions (Wherry and Ahmed, 2004; Wherry et al., 2003). How these subsets of cells arise is still a subject of some controversy (Chang et al., 2007). At the time that this thesis was initiated, little was known about $CD8^+$ T cell memory development during parasitic infection, and how that compared to what has been studied in viral systems.

It has been known for many years that T cells from chronically infected mice confer protection upon adoptive transfer (Parker et al., 1991) or virulent challenge (Gazzinelli et al., 1991), but the phenotype and function of the cells mediating this protection had not been closely studied. Previous examination of the memory responses induced by ts-4 were complicated by the fact that this strain can continue to replicate and cause disease in immunodeficient hosts (Sayles and Johnson, 1996). The CPS-OVA parasites enabled study of the antigen-specific cells and while OVA-specific cells were easily detectable in the blood, bone marrow or spleen, they were difficult to detect in the LN. For the most part, these OVA-specific $CD8^+$ T cells still resembled effector memory cells ($KLRG1^+CD62L$-), even 3 months following immunization. I did see that a small proportion (10-20%) of OVA-specific cells had high levels of CD62L and low levels of

KLRG1, a phenotype typically associated with central memory T cells, though approximately two-thirds were expressing high levels of IL-7Rα (Chapter 3). Further studies are required to determine whether or not these cells had functional differences compared to $CD8^+$ T cells with a similar phenotype that were generated in response to viral or bacterial challenge.

As discussed previously, numerous studies have indicated that the pro-inflammatory cytokine IL-12 plays a key role in the response to *T. gondii* (Gazzinelli et al., 1993; Gazzinelli et al., 1994; Hunter et al., 1995b; Khan et al., 1994). Evidence continues to mount that this cytokine is also integral in the regulation of $CD8^+$ T cell memory and effector differentiation, with high levels of IL-12 correlated with the preferential formation of effector cells but a poor memory pool (Joshi et al., 2007; Wilson et al., 2008). While my work very clearly implicates IL-12 in the initial expansion of parasite-specific $CD8^+$ T cells, there was only correlative evidence that IL-12 levels influenced memory generation to CPS-OVA. This evidence rests largely on two findings. First, the observation that c-$Rel^{-/-}$ OVA-specific $CD8^+$ T cells expressed higher levels of CD127 and CD27, both markers that have been associated with memory formation in other models. Additionally, the c-$Rel^{-/-}$ OVA-specific $CD8^+$ T cells responded better to secondary challenge as compared to the WT cells by expanding to a greater degree. This second finding suggests that c-Rel is not required for proliferation of memory T cells, and provides one potential mechanism whereby c-$Rel^{-/-}$ mice could generate better $CD8^+$ T cell memory based on their primary expansion in a low IL-12 environment (Figure 28). Other cytokines such as IL-2 have also been shown to contribute to memory $CD8^+$ T cell development and so the role of additional cytokines or growth factors cannot be

discounted (Kalia et al., 2010; Pipkin et al., 2010; Williams et al., 2006), but were not examined in the course of this thesis work.

Another important direction for the field of immunity to infection is to gain better understanding of the transcription factors that regulate developmental pathways of $CD8^+$ T cells and their differentiation into effector and memory cells. For example, my work with CPS-OVA parasites, in agreement with an expanding literature, suggests that IL-12 levels are a critical regulator of $CD8^+$ T cell expansion as well as memory formation. IL-12 has been shown by a number of others to regulate expression of the transcription factors T-bet and Eomesodermin (Takemoto et al., 2006), a pathway which recent work has demonstrated is likely dependent on the mTor kinase (Araki et al., 2009; Rao et al., 2010). While c-Rel does not seem to play a $CD8^+$ T cell-intrinsic role in the studies presented here, its importance in regulatory T cells might be exploited therapeutically to make existing vaccines, or responses of memory cells upon challenge, more effective. Full understanding and more complete identification of the downstream targets of T cell-specific transcriptions factors, and the protective effector functions induced following their activation, could make drug treatment or vaccines work more effectively. In summary, the work presented in this thesis has examined some of the factors required for generation of an optimal $CD8^+$ T cell response, and formed the basis for on-going studies related to $CD8^+$ T cell memory. Further, these studies have demonstrated the importance of the inflammatory environment and the contributions of T cell-extrinsic factors to the generation of adaptive immune responses to *T. gondii*. Better understanding of these factors will be important not only to further understanding of the biology of parasite

infection, but will also inform future research into vaccines or treatments against pathogens that affect human health and disease.

Figure 28. Model for CD8$^+$ T cell development following *T. gondii* infection

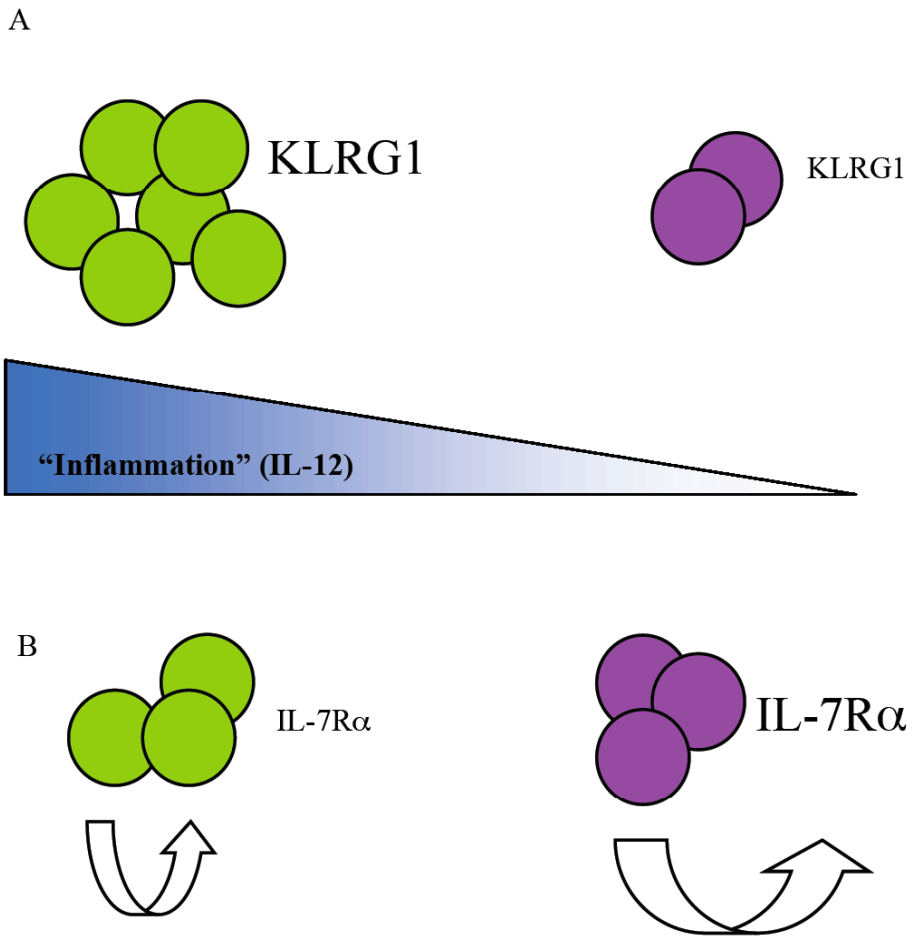

Figure 28. Levels of inflammation, exemplified throughout these thesis studies by IL-12, influence effector versus memory formation of antigen-specific CD8$^+$ T cells. Different factors, including cytokines, antigen exposure, and CD4$^+$ T cell help, act to promote this maturation. (A) During the effector stage, IL-12 controls expansion and KLRG1 expression on antigen-specific CD8$^+$ T cells. but which express higher levels of IL-7Rα during the memory phase. (B) Cells generated in the inflammatory environments as represented in (A) can still be maintained at similar levels but express varying levels of IL-7Rα, and may respond differently to secondary challenge (expansion represented by the differently-sized arrows).

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